A WATERHEMP SAGA: SEED PRODUCTION, GENETICS, HYBRIDIZATION, AND THE CREATION AND DISCOVERY OF QUAD-STACK INDIVIDUALS

BY

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THESIS

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ABSTRACT

Waterhemp is an old weed posing new problems for corn and soybean producers in the midwestern United States. The weed is indigenous to the Midwest, but has only become a major problem within the last two decades, and it is now one of the most prevalent weeds in Illinois. One of the most troubling aspects of this species is its propensity to evolve resistance to herbicides—a feat which it has now accomplished for herbicides with four different modes of action, with the evolution of resistance to other herbicide modes of action expected in the future. Options for chemical control of this species—particularly for postemergence control in soybean—are rapidly declining. In fact, of the four herbicide chemistries currently available for broadleaf control in soybean, some waterhemp populations have evolved resistance to three. This thesis addresses several facets of this fascinating species, beginning with a literature review in Chapter 1 on the history of weed control, some background on how weeds evolve resistance to herbicides, the biology of waterhemp, and the evolution of herbicide resistance within waterhemp specifically. Chapter 2 addresses a study on an aspect of the reproductive biology of waterhemp—namely the amount of time required for female plants to produce mature seeds after pollination. It was found that some seeds may become viable in as little as 7–9 days after pollination, and that seed dormancy drops if seeds remain on the plant for at least 15–30 days after pollination. These findings could be helpful in future studies requiring the crossing of waterhemp, such as the study reported in Chapter 3, in which the inheritance and genetics of glyphosate resistance in a Missouri waterhemp population are investigated. Glyphosate resistance was determined to be a nuclear-inherited dominant or partially dominant trait, although the number of genes involved could not be determined. Investigations into gene

amplification of *EPSPS*, which has been shown to confer glyphosate resistance in the related species, Palmer amaranth, did reveal elevated copy number in the Missouri population. However, analysis of copy number in F_1 and F_2 populations showed that copy number does not strictly cosegregate with resistance level, indicating that at least one other factor is necessary for resistance. Several of the F_2 populations created for the study in Chapter 3 (involving the crossing of a population resistant to ALS inhibitors, PPO inhibitors and PS II inhibitors with the glyphosate-resistant Missouri population) were investigated in Chapter 4 for the presence of four types of resistance, and individual plants were identified containing all four resistance types, indicating no significant barriers to the combination of four herbicide resistance types within a single plant. Further studies showed tight linkage between ALS and PPO resistance, but no linkage among other types of resistance was detected. In Chapter 5, two waterhemp populations collected from fields in Illinois are examined for multiple herbicide resistance. One population was found to be resistant to glyphosate as well as ALS inhibitors, and the other population was found to be resistant to glyphosate, ALS inhibitors, PPO inhibitors, and PS II inhibitors. Individuals from this population were also identified as being four-way resistant, thus confirming what was observed in greenhouse experiments in the previous chapter. Chapter 6 addresses an attempt at transferring glyphosate resistance from plants of the Missouri waterhemp population into smooth pigweed through hybridization. Progeny were confirmed as hybrids by use of ITS markers, and hybrid plants were found to be resistant to glyphosate. Hybrids were backcrossed (BC) to smooth pigweed, but produced very few seed, preventing the screening of the BC progeny. The BC progeny were again backcrossed to smooth pigweed and found to segregate for seed production, although little seed was produced overall. Although incomplete, this study

suggests that such transferal of glyphosate resistance in nature is unlikely. Finally, Chapter 7 discusses concluding remarks, implications and future research.

To Mom and Dad—
May your pastures always be weed-free.
But if they're not, may 2,4-D always kill the thistles.

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CHAPTER 1

INTRODUCTION

1.1 A Brief History of Weed Control

The term "weed" may, at first glance, seem relatively easy to define. Undoubtedly, everyone will at least agree that a weed is a plant. Beyond that, however, one may encounter some discordant perceptions on which species qualify as weeds. The truth is that what one person would not hesitate to call a weed may in fact be, to another person, a valuable plant. For instance, an oak seedling growing in a hayfield would likely be considered a weed by the farmer concerned with harvesting enough hay to feed his livestock over the winter, and he will probably remove it before it becomes large enough to damage his mower or to seriously compete with the clover in his field. Meanwhile a woman that recently built a house nearby may be considering purchasing an oak seedling to plant in her yard to eventually provide shade, after having just placed a container of herbicide in her shopping cart that she plans to use to control that pesky clover that always seems to take the place of her grass. Thus, it appears that people may have differing views on which plants they consider to be weeds, but from the above example, one may begin to infer that a weed is an unwanted plant. A more descriptive and entertaining definition of a weed is given by Clark and Fletcher, in their 1906 book titled "Farm Weeds of Canada" where they define a weed as "any injurious, troublesome, or unsightly plant that is at the same time useless or comparatively so." Which plant species qualify as weeds under this definition obviously depends on the person doing the defining.

Although early evidence of humans' awareness of weeds as pests is anecdotal at best (Zimdahl 2010), humans have likely been growing crops alongside weeds ever since the advent

of farming, which is believed to have occurred, at least in a primitive form, sometime between 15,000 and 10,000 B.C. (Wells 1961). In his 1970 *Weed Science* article, "A History of Weed Control in the United States and Canada," F. L. Timmons provides a detailed review of the evolution of weed control from the use of the earliest tools consisting of bone or wood, through the weed control strategies that are still used today. A brief outline of his article follows.

Although evidence exists of humans developing tools for planting and harvesting crops by as early as 3000 B.C., Timmons claims that the use of such tools for weed control was probably rare. By the first century A.D. it seems that some humans understood the negative impacts that weeds can have on crop production, as an early Roman writer noted that yields were reduced if weeds were not removed from a field. Some early British publications also exist that show that humans had begun to understand the ill-effects of weeds on crops. Timmons writes that a law was enforced in Scotland from 1212 to 1249 that severely penalized tenants or servants for sowing weeds or for not destroying certain weeds. However, Timmons states that the overarching philosophy at the time was probably that weeds were an unavoidable nuisance and that little besides hand-weeding could be done to keep them in check.

Some progress in mechanical weed control came with the development of farming implements beginning around the 1600s. By 1777 the plow was apparently an important tool for weed control in Sweden. Horse-drawn steel-shovel and straddle row cultivators were invented in the mid-1800s, which marked an important advancement in weed control. Advancements such as the development of the gasoline tractor continued, and by 1925 developments in mechanical weed control were occurring rapidly.

The early 1940s marked the beginning of a revolution in weed control, which Timmons refers to as the beginning of the "Chemical Era of Agriculture" with the development of the

phenoxyacetic acid herbicides. However, this was not the first use of chemicals in agriculture, as Timmons states that even as early as 1000 B.C. humans used sulfur for pest control. Salt was recommended for use as an herbicide in Germany in 1840, although Timmons writes that it was probably used much earlier.

Through the late 1800s and early 1900s, numerous compounds were tested and used as herbicides with limited success, as treatment with these compounds generally required large quantities of herbicide, resulting in a high cost per acre. These compounds were also frequently toxic, flammable or corrosive and rarely provided consistent weed control, preventing them from gaining popularity (Peterson 1967). However, with the creation of the phenoxyacetic acid herbicides (discussed in detail by G. E. Peterson in his 1963 *Agrictultural History* article, "The Discovery and Development of 2,4-D"), weed control would be forever changed.

The public first became excited about these herbicides—particularly 2,4-dichlorophenoxyacetic acid (2,4-D)—after hearing a 1944 report that bindweed (Convolvulaceae family), a troublesome weed for farmers, could be killed within 10 days of being sprayed with this herbicide (Peterson 1967). Results of a study conducted almost simultaneously showed that 2,4-D could provide a complete kill of dandelion [*Taraxacum officinale* G. H. Webber ex Wiggers] within 18 days, and that, when sprayed on turf, this herbicide killed clover (*Trifolium* spp.) while leaving bluegrass [*Poa annua* L.] unharmed (Mitchell et al. 1944). Subsequent toxicity tests showed that the herbicide was safe, with one somewhat amusing report of E. J. Kraus, Head of the Botany Department at the University of Chicago at the time, claiming that he had even ingested 0.5 g of 2,4-D daily for three weeks and had experienced no effects whatsoever (Peterson 1967). With such reports of the effectiveness and low mammalian toxicity of this herbicide, some chemical companies decided to begin marketing 2,4-D in 1945, and thus

mass-production of the herbicide began. In the first year that 2,4-D was on the market, 917,000 pounds of this product were produced in the United States (US). In the following year, production jumped to 5,466,000 pounds. The price also dropped drastically from $12.50 per pound in 1945 to under $3.00 per pound one year later, and by 1950 the price had dropped to $0.50 per pound (Peterson 1967). With the introduction of 2,4-D into the market, farmers were now able to apply an herbicide to their fields that was cheap, safe, and that effectively killed many broadleaf weed species. This success in chemical weed control opened the door for the production of other types of herbicides and by 1949 chemical companies were marketing 20 different types of organic herbicides (Peterson 1967).

Since those early days of effective chemical weed control, the number of herbicides on the market has greatly increased, due in part to the discovery of new herbicidal compounds as well as the creation of new formulations of old compounds. Chemical weed control is still a very popular practice for farmers. However, with this major advancement in weed control have come some other, perhaps originally unforeseen, negative consequences.

1.2 Evolution on a Short Time-Scale

In his revolutionary work "On the Origin of Species," Charles Darwin proposed that all forms of life have reached their current state through evolution, which he claims is driven by natural selection (1859). Darwin defines natural selection as "[the] preservation of favourable individual differences and variations, and the destruction of those which are injurious…" (1859). He goes on to say that evolution is a continuous process and is still occurring today, writing that "It may be said that natural selection is daily and hourly scrutinising, throughout the world, every variation, even the slightest; rejecting that which is bad, preserving and adding up all that is

good; silently and insensibly working, whenever and wherever opportunity offers, at the improvement of each organic being in relation to its organic and inorganic conditions of life" (1859). In few disciplines is the truth of this statement as obvious as it is in weed science with the evolution of herbicide-resistant weeds.

The basic idea of evolution through natural selection can be succinctly summarized as the "survival of the fittest." In other words, individuals that are most fit for a certain environment (i.e., the fittest) will thrive and produce a relatively large number of offspring when compared with less fit individuals, which in some cases may not even live long enough to reproduce. In other cases there may be a nearly undetectable (but still present) difference in the fitness levels of individuals, and the evolution of a species into the fittest form may occur over centuries or millennia. However, just as the definition of a weed was somewhat ambiguous in that the specific plants which are considered as weeds depends on the person doing the defining, so too does the definition of the "fittest" depend on the environment which is being considered. For instance, an animal with an efficient method of dissipating body heat would likely be well-suited for living in a tropical region. However, this same animal may not be well-suited for life in the Arctic, where insulation for the retention of body heat is probably a more important trait than is that of efficient heat dissipation. Thus, the "fittest" is obviously a relative term, with the definition depending on what kinds of selection pressure are being applied by a particular environment to the life forms living there.

The environment in agronomic cropping systems today is a harsh one for weeds—especially in fields in which chemical weed control is practiced. This is due to the fact that contemporary herbicides are highly efficacious, with most killing at least 90% of the susceptible weeds in a field and therefore applying intense selection on the weeds in such fields (Jasieniuk et

al. 1996). Although the selection pressure imposed on weeds by these herbicides may not be exactly "natural" per se, it is selection nonetheless, and this selection has created some very troubling issues when it comes to chemical control of certain weed species—namely the evolution of herbicide-resistant weeds. As the majority of this thesis deals with herbicide resistance in weeds, it may be worthwhile to briefly delve into how the evolution of such resistance to herbicides occurs, beginning with a general look at how herbicides kill plants.

Herbicides are often grouped into families based on the chemical structure of the active ingredient. Another way to classify herbicides is by their mode of action and site of action. The mode of action of an herbicide is related to the way the chemical kills a plant. For instance, some herbicides work by indirectly causing plants to produce free radicals in the presence of light, which ultimately destroy cell membranes through lipid peroxidation and eventually cause plant death. Such herbicides may be referred to as light-activated herbicides (Hess 2000). This method of causing the death of a plant would be considered the mode of action of the herbicide.

The site of action of an herbicide is different from the mode of action, and understanding an herbicide's site of action is a good place to begin to understand a common method by which plants evolve resistance to herbicides. The site of action refers to the site in a plant cell that is directly affected by an herbicide, usually referring to a specific enzyme to which an herbicide binds, thereafter inhibiting the normal function of the enzyme and leading to the demise of the plant. In this case, the enzyme inhibited by the herbicide would be the herbicide's site of action.

A common mechanism by which weeds survive herbicide treatment and become classified as herbicide-resistant is through a modification of the site of action. Such modifications may successfully confer resistance to an herbicide when they preserve functionality of the enzyme but interfere with the inhibitory effect of the herbicide. This often

amounts to a change of an amino acid located at or near the specific region where the herbicide binds to the enzyme (i.e., the target site). However, this is not the only resistance mechanism exhibited by plants. Some other resistance mechanisms include reduced uptake of herbicide (Schulz et al. 1990), reduced translocation of herbicide throughout the plant (Powles and Preston 2006), and metabolism of the herbicide into nontoxic compounds within plant cells (Gronwald et al. 1989), to name a few. These resistance mechanisms work by preventing the herbicide from reaching the site of action.

At first glance this may almost seem like magic. How can plants learn to modify their own enzymes to survive treatment with an herbicide? How can they learn to metabolize herbicidal compounds, or to prevent herbicides from reaching their sites of action in the first place? However, the truth is that weeds do not "learn" to become resistant to herbicides. Instead, naturally-occurring resistant individuals are sometimes present in a weed population, and when herbicides are applied to such a population the susceptible plants are killed, while the resistant plants survive. In other words, the herbicide "selects" for the resistant biotype—the fittest plants in an environment in which herbicides are present. In some cases these resistant plants may survive long enough to produce seeds. After several applications of the same herbicide on such a population, the frequency of resistant individuals may begin to increase, eventually leading to a population becoming herbicide-resistant. For herbicide resistance to become a reality, however, two important factors must be present.

One essential ingredient in the evolution of herbicide resistance in weeds is the presence of genetic diversity in the weed species. If no naturally-occurring herbicide-resistant individuals are present in a weed population, there is no chance for the weeds to evolve resistance to the herbicide. Or, worded differently, naturally-occurring resistant individuals can only appear in a

susceptible population if sufficient genetic diversity exists in the weed population. Genetic diversity may be acquired in a weed species in several ways, including spontaneous mutation, meiotic recombination, DNA replication errors, or interspecific hybridization (Jasieniuk et al. 1996; Trucco et al. 2005a). In particular, spontaneous mutations at gene loci are thought to occur with characteristic frequency—typically between 1×10^{-5} and 1×10^{-6} gametes per locus per generation—leading to new mutations being continuously produced in plant populations (Jasieniuk et al. 1996; Hedrick 2005). If these mutations occur in genes encoding enzymes which happen to be the site of action of a particular herbicide, some of these mutations may confer resistance to the herbicide. Such a resistance-conferring mutation would be considered a target-site mutation.

Another essential ingredient in the evolution of herbicide resistance in weeds is that of selection pressure applied to the weed population by the herbicide. No matter how much natural genetic diversity is present in an herbicide-susceptible weed population, without the application of herbicides, resistant individuals will not be selected for survival in the field. This may be best understood by again considering evolution as the survival of the fittest. In the absence of herbicide, the fittest weeds are likely those herbicide-susceptible plants that are just like the majority of the others present in the field, hereafter referred to as the wild type. The wild type represents a weed biotype which has evolved to its current state through natural selection as proposed by Darwin. In theory, this biotype should represent the fittest form of a particular species in a given environment. Presumably, with static environmental conditions, any changes in the genotype of such plants, including those that could confer resistance to an herbicide, should be at least slightly detrimental, for if they were instead beneficial, the wild type would likely already possess such a genotype.

However, with the application of an herbicide, the environment in the field changes drastically. What was previously the fittest genotype of an herbicide-susceptible species—the wild type—is no longer the fittest. In fact, according to Jasieniuk et al. (1996), one may expect the vast majority of such plants to be killed by the herbicide. With the change in environment then, comes a change in the genotype of plants which are the best suited for survival in that environment. In this case, plants with an herbicide-resistant genotype become the fittest, as these are the plants that will survive treatment with herbicide and that may eventually produce seed. Over time, if the same herbicide is repeatedly applied, this population may eventually become an herbicide-resistant weed population (Jasieniuk et al. 1996). Thus, along with sufficient genetic diversity in a weed species, herbicide selection pressure is also necessary for weeds to evolve resistance to herbicides.

In recent years there has been no shortage of examples of weed species for which both of these important factors are present, and herbicide resistance is now a serious issue in agriculture worldwide (Heap 2010). The first weed to evolve resistance to an herbicide was spreading dayflower [*Commelina diffusa* Burm. f.], which was reported resistant to 2,4-D in 1957, surviving a rate five times higher than that which controlled susceptible plants (University of Idaho 2007; Heap 2010). Since then, many more weeds have evolved resistance to herbicides, which may be attributable to the production and marketing of numerous other herbicidal compounds. At last count, 346 biotypes out of a total of 194 weed species have evolved herbicide resistance (Heap 2010), and this number will continue to grow. However, although many weeds have evolved resistance to herbicides around the world, weeds of one genus in particular—the *Amaranthus* genus—pose a serious threat to corn [*Zea mays* L.] and soybean [*Glycine max* (L.) Merr.] producers in the Midwestern US. Of the species in this genus, one has

been especially problematic. That species is waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer].

1.3 Waterhemp Biology

The *Amaranthus* genus consists of about 70 species worldwide (Robertson 1981; Costea et al. 2005), at least 10 of which are present as major weeds in the U.S., including smooth pigweed [*A. hybridus* L.], redroot pigweed [*A. retroflexus* L.], Palmer amaranth [*A. palmeri* S. Wats.], and waterhemp (Great Plains Flora Association 1986; Horak et al. 1994; Wax 1995). Of these ten species, smooth pigweed and waterhemp have been listed as among the most prevalent weeds in Illinois (Wax 1995; Hager et al. 2002). Several factors have contributed to this listing—perhaps the most important of which is the ability of these species to evolve resistance to herbicides (Heap 2010). Waterhemp is a particularly troublesome species, as it has evolved resistance to herbicides representing four different modes of action within the past two decades (Heap 2010). However, before investigating herbicide resistance in this species, it may be instructive to study the characteristics that originally contributed to the success currently realized by this weed.

Waterhemp is a small-seeded summer annual, meaning that it completes its life-cycle within a single summer growing season. It is a C_4 broadleaf plant, allowing for rapid growth in high-light and high-temperature environments. Such plants are also better able to tolerate drought and to continue photosynthesis under water stress when compared with C_3 species growing under similar conditions (Hopkins and Hüner 2004). Under ideal growing conditions and in the absence of competition with other plants, waterhemp may grow to more than 2 m tall (Horak and Loughin 2000; Costea et al. 2005) and may produce in excess of 1×10^6 seeds per

plant (Steckel et al. 2003). These seeds are persistent in the soil seed bank, with 11% of such seeds remaining viable after 4 years of burial (Buhler and Hartzler 2001), and 1–3% of these seeds capable of germination even after 17 years of burial at a depth of 20 cm (Burnside et al. 1996). With seed production of at least hundreds of thousands per plant, this amounts to thousands of seeds from a particular waterhemp plant remaining viable for nearly two decades!

Waterhemp is dioecious, meaning that the species consists of male plants, which produce only pollen, and of female plants, which produce only seeds. Thus, this species is an obligate outcrosser, as pollen must travel from male to female plants for seed production to occur. This species is wind-pollinated (Murray 1940), and the pollen has been predicted to be capable of traveling significant distances from the male plants. In fact, Costea et al. (2005) predict that airborne waterhemp pollen may travel at least 300 m from a 1 m tall male plant in wind speeds of 40 km h^{-1}. Thus, a female plant may potentially be pollinated by many different male plants, which contributes to the high level of genetic diversity in this species (Hager et al. 1997).

Further contributing to the genetic diversity of waterhemp is its ability to hybridize with other species in the *Amaranthus* genus (Trucco et al. 2005a; Murray 1940; Sauer 1957). Ellstrand and Schierenbeck (2000) have suggested that hybrid-derived plant populations contain much more genetic variation than do the parental species. Numerous studies have shown the potential for waterhemp to hybridize with smooth pigweed (Trucco et al. 2005a, 2005b; Tranel et al. 2002) and with Palmer amaranth (Franssen et al. 2001, Wetzel et al. 1999). Therefore, hybridization may serve as a significant source of genetic diversity in waterhemp.

Although waterhemp is indigenous to North America (Sauer 1955), it has only become a major problem weed within the last 20 years (Hager et al. 2002). Originally found predominantly near riverbanks and lakeshores or pond margins (Sauer 1957), waterhemp now infests many

fields throughout the midwestern US (Trucco et al. 2006). This change in habitat may be attributed to several factors. One such factor is the relatively recent shift toward no-tillage or reduced-tillage practices in agronomic cropping systems. Because waterhemp seeds are small, they tend to germinate and grow best when they remain near the soil surface (Buhler 1992; Hager et al. 1997). No-tillage practices allow seeds to remain undisturbed near the soil surface, whereas tillage tends to bury seeds so that germination of small seeds is greatly reduced. These seeds also exhibit varying levels of dormancy, which is yet another factor contributing to the recent success of waterhemp as a weed in agronomic cropping systems. Seed dormancy not only contributes to the longevity of waterhemp seeds in the soil seed bank (Leon et al. 2007), but it also causes a tendency for waterhemp to germinate throughout the growing season (Allen and Meyer 1998), making season-long control following a single herbicide application difficult (Hager et al. 1997). When taken at face value, these factors alone are enough to make waterhemp a formidable weed, allowing it to effectively compete with crops for limited resources such as light, water, and nutrients. However, it is the interaction of these factors that has afforded waterhemp the ability to survive treatment with herbicides that were once quite effective in controlling this species.

1.4 Herbicide Resistance in Waterhemp

Waterhemp's ability to quickly evolve resistance to herbicides is likely due to the interaction of its high seed production with the variable levels of dormancy within these seeds. The high seed production of this species increases the probability that at least some of the seeds produced will contain resistance-conferring mutations in their DNA (Jasieniuk et al. 1996). As was noted earlier, however, these resistance conferring mutations alone will not lead to an

herbicide-resistant weed population without selection pressure from herbicides. This is where seed dormancy plays an important role.

It has been noted that the variable levels of dormancy in waterhemp seeds causes the seeds to germinate throughout much of the growing season, which in turn means that achieving season-long control of this species following a single herbicide application is unlikely. While soil-applied herbicides may control waterhemp early in the growing season, these herbicides may not be persistent enough in the soil to control waterhemp germinating later in the season. Likewise, postemergence herbicides must be applied to small seedlings to be effective, and few have sufficient residual activity to control later-emerging seedlings (Hager et al. 1997). Thus, in order to achieve acceptable season-long control of this species with herbicides, multiple treatments are often needed. However, more herbicide applications means higher selection pressure for resistant individuals (Jasieniuk et al. 1996). (This is particularly true with waterhemp, which often exhibits very high seedling densities shortly after germination. During growth, many of the weakest plants necessarily die due to competition before herbicide application. Thus, by germinating in several flushes throughout the growing season, rather than all at once, more seedlings may be exposed to selection from herbicides.) When this high selection pressure is combined with the high seed production and genetic diversity in this species, waterhemp becomes a prime candidate for rapidly evolving resistance to herbicides. This is precisely what has been observed in the past 20 years, with waterhemp evolving resistance to herbicides representing four modes of action—triazines, acetolactate synthase (ALS)-inhibitors, protoporphyrinogen oxidase (PPO)-inhibitors, and glyphosate.

Waterhemp first evolved resistance to ALS-inhibiting herbicides in Illinois and Iowa in 1993 (Heap 2010). ALS-inhibitor resistance was followed by the evolution of triazine-resistant

waterhemp in Missouri in 1994. Soon afterward, in 1996, the first waterhemp population with resistance to multiple herbicide modes of action was identified in Illinois (Foes et al. 1998). This population demonstrated resistance to both ALS-inhibitors and triazines. In 2001 a waterhemp population was identified in Kansas, which contained individuals resistant to both PPO-inhibitors and ALS-inhibitors, which was followed a year later by the identification of a population in Illinois which demonstrated resistance to ALS- and PPO-inhibitors and triazines—the world's first 3-way resistant waterhemp population (Patzoldt et al. 2005). Glyphosate resistance was identified in waterhemp in a Missouri population in 2005, which also demonstrated resistance to ALS- and PPO-inhibiting herbicides (Legleiter and Bradley 2008). In the following sections, each of these herbicides and resistance types are investigated in detail.

1.4.1 Triazine resistance

Triazines—members of a larger class of photosystem II (PS II)-inhibiting herbicides—work by competing with plastoquinone (Q_B) for its binding site on the D1 protein in the PS II pathway (Hess 2000). This inhibition blocks electron flow through the pathway, which ultimately leads to the production of singlet oxygen, leading to lipid peroxidation and causing plant cells to leak their contents and die (Hess 2000). These herbicides have been used since 1956 (Patzoldt et al. 2003), and they remain popular due to their broad-spectrum weed control and soil-residual activity (Maertens et al. 2004). However, their popularity has meant strong selection pressure for resistant weed biotypes, and populations of at least 68 species have now been identified as resistant to this class of herbicide (Heap 2010).

Resistance to PS II inhibitors—specifically to triazines—can be conferred via several mechanisms. One mechanism is a mutation in the chloroplastic *psbA* gene, which encodes the D1

protein (Foes et al. 1999). Mutations in this gene can alter the target site (the Q_B binding site) of the protein, thereby eliminating the herbicides' affinity for the target site. Specifically, such mutations cause a substitution of glycine for serine at amino acid residue 264 of the D1 protein (Gronwald 1994; Foes et al. 1998, 1999). This substitution eliminates competition between Q_B and the herbicide, which allows the plant to survive treatment with such herbicides. Because target site resistance to triazines occurs due to a mutation in a gene found in the chloroplast, this type of resistance is a maternally-inherited trait. However, another mechanism of resistance to triazine herbicides has been documented in velvetleaf [*Abutilon theophrasti* Medik.], which consists of plants metabolizing and thus detoxifying the herbicides (Gronwald et al. 1989). This type of resistance is nuclear-inherited and therefore may be spread through pollen flow (Gronwald et al. 1989). This has been suggested as a possible resistance mechanism in waterhemp (Patzoldt et al. 2003) in addition to the target site mutation in *psbA* that has been observed in some biotypes (Foes et al. 1998).

1.4.2 ALS-inhibitor resistance

ALS-inhibiting herbicides control weeds by blocking the production of branched-chain amino acids in plants, thereby effectively starving plants to death (Tranel and Wright 2002). These herbicides initially gained popularity in the 1980s, mainly due to the fact that even at very low use rates they controlled a broad spectrum of weeds and they had some residual activity in the soil while having relatively low crop and mammalian toxicities (Tranel and Wright 2002).

The convenience in weed control provided by these herbicides led to their being widely used, resulting in heavy selection pressure for ALS-resistant weed biotypes. The first weed species to evolve resistance to ALS-inhibiting herbicides was prickly lettuce [*Lactuca serriola*

L.] identified in 1987 (Mallory-Smith et al. 1990). Since then, 106 other species have evolved resistance to these herbicides (Heap 2010). Past research has shown that mutations conferring resistance to these herbicides are fairly common. In fact, at least six different naturally-occurring mutations in *ALS* are known to confer target-site resistance to these herbicides (Tranel and Wright 2002; Whaley et al. 2007) with little or no known fitness penalties (Holt and Thill 1994). Target-site ALS-resistance is a dominant, nuclear-inherited trait which may be spread easily via pollen flow (Tranel and Wright 2002), particularly in outcrossing species such as waterhemp. A survey of Illinois waterhemp conducted by Pazoldt and Tranel (2007) indicated that at least three mutations in *ALS* may confer resistance to ALS-inhibiting herbicides in this species. One of these mutations consists of the substitution of leucine for tryptophan at amino acid position 574 (W574L) in ALS. The other two mutations occurred at amino acid position 653, with either asparagine or threonine being substituted for serine (written as S653N or S653T, respectively). Target-site ALS-resistance in waterhemp is so common today that these herbicides are no longer recommended for control of this species (Hager and Sprague 2003; Sprague et al. 1997).

1.4.3 PPO-inhibitor resistance

PPO-inhibiting herbicides are members of a larger class of light-dependent herbicides (Hess 2000). These herbicides work by binding to the protoporphyrinogen oxidase (Protox) enzyme in the plastid membrane, thereby effectively blocking the last step of the heme and chlorophyll biosynthesis pathway (Beale and Weinstein 1990). This enzyme normally catalyzes the transformation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX), but when the herbicide blocks Protox, the precursor molecule (Protogen IX) accumulates in the chloroplast and begins to leak out into the cytoplasm. There it is converted via an unknown

mechanism to Proto IX, which reacts with oxygen and light to produce singlet oxygen. The singlet oxygen then reacts with lipids in the cell membrane leading to lipid peroxidation and ultimately resulting in the destruction of cell membranes and causing plant death (Duke et al. 1991).

PPO-inhibitors may be soil-applied or foliar-applied, with the latter being fast-acting, contact-burning herbicides, which have been popular and effective tools for broadleaf weed control for at least 30 years (Li et al. 2004) and have been marketed since as early as the 1960s (Matsunaka 1976). They have been particularly useful in controlling these weeds in conventional soybean fields (Li et al. 2004). Despite their long use, it has been only recently that weeds resistant to these herbicides have been identified. In fact, the first weed identified as being resistant to PPO-inhibiting herbicides was waterhemp in 2001, and to date only three other species (*Amaranthus quitensis, Ambrosia artemisiifolia,* and *Euphorbia heterophylla*) have evolved resistance to this class of herbicide (Heap 2010).

Resistance to PPO-inhibitors is conferred by a loss of three nucleotides in the Protox-encoding gene, *PPX2,* which results in the deletion of an amino acid (glycine) at codon 210—a mutation commonly written as ΔG210 (Patzoldt et al. 2006). This position is located near the herbicide-binding site of Protox, and the mutation confers resistance to all three families of PPO-inhibiting herbicides (Patzoldt et al. 2005). This is the first codon deletion implicated in conferring resistance to an herbicide, and to date this is the only mechanism known to confer resistance to PPO-inhibiting herbicides in waterhemp (Lee et al. 2008).

1.4.4 Glyphosate resistance

Glyphosate is a broad-spectrum herbicide, controlling many annual and perennial grasses and broadleaf plants, that has been used extensively worldwide since its introduction in 1974 (Baylis 2000). In fact, glyphosate is the most widely used herbicide in the world (Preston and Wakelin 2008). Glyphosate controls weeds by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which plays a key role in aromatic amino acid biosynthesis (Bradshaw et al. 1997). Thus, when a plant is treated with glyphosate it can no longer produce proteins, leading to the demise of the plant. This herbicide is effective on perennials because it is a systemic herbicide—after entering the plant (usually through the leaves), glyphosate is translocated throughout the plant including to the roots, moving primarily through the phloem (Franz et al. 1997).

Even as recently as ten years ago, it appeared, due to this herbicide's high efficacy combined with widespread use over at least a 20-year interval during which there was a total lack of examples of weeds that had evolved resistance to this herbicide, that the probability of weeds evolving resistance to glyphosate in the future was low (Bradshaw et al. 1997). Also involved in making this prediction was the difficulty experienced in attempting to produce glyphosate-resistant crops (Bradshaw et al. 1997). However, in 1996 Monsanto began marketing glyphosate-resistant (Roundup-Ready®) soybeans, and glyphosate use in soybean increased dramatically from 2.5 to 30 million kg yr^{-1} (Young 2006). Farmers using this technology could apply glyphosate to their soybeans to control nearly every weed in their fields without harming the crop. This provided a very convenient and effective method of weed control.

Since 1996, other glyphosate-resistant crops have been introduced, including corn and cotton [*Gossypium hirsutum* L.], and by 2006, approximately 80 x 10^6 ha around the world were

planted with glyphosate-resistant crops (James 2006). However, the ease with which weeds could be controlled with glyphosate in such cropping systems quickly led to overreliance on this herbicide, which in turn led to higher selection pressure for glyphosate resistant-weeds. Thus it was only a matter of time until glyphosate-resistant weeds evolved.

The first species reported resistant to glyphosate was rigid ryegrass [*Lolium rigidum* Gaudin] in 1996 in a field that had received glyphosate treatments for the previous 15 years (Pratley et al. 1999). Since 1996 at least 19 other species have evolved resistance to this herbicide (Heap 2010), including waterhemp (Legleiter and Bradley 2008). The mechanisms of resistance to glyphosate have been more difficult to elucidate than have the mechanisms for other types of herbicide resistance. In fact, the glyphosate resistance mechanism in waterhemp remains unknown. However, to date three mechanisms have been discovered that confer resistance to glyphosate in other weed species. One such mechanism is reduced translocation of glyphosate from the leaves to the rest of the plant. This mechanism has been implicated in conferring glyphosate resistance in rigid ryegrass (Lorraine-Colwill et al. 2003), Italian ryegrass [*Lolium multiflorum* (Lam.) Husnot] (Michette et al. 2005), and horseweed [*Conyza canadensis* (L.) Cronq.] (Feng et al. 2004). Target-site mutations at position 106 in EPSPS in which a proline is replaced by either serine or threonine (P106S or P106T, respectively) have also been implicated in conferring resistance in goosegrass [*Eleusine indica* (L.) Gaertn.] (Baerson et al. 2002) and rigid ryegrass (Wakelin and Preston 2006). Both of these resistance mechanisms are nuclear-inherited single-gene traits (Powles and Preston 2006). Interestingly, the reduced translocation mechanism confers a higher level of resistance to glyphosate (7- to 11-fold) than does the target site mutation, which provides a 2- to 3-fold increase in resistance level when compared with susceptible populations (Preston and Wakelin 2008; Wakelin and Preston 2006).

Recently yet another mechanism that confers resistance to glyphosate has been discovered from studies conducted on glyphosate-resistant Palmer amaranth. The resistance mechanism in this species has been identified as gene amplification of *EPSPS* and increased expression of EPSPS (Gaines et al. 2010), and this mechanism conferred a 6 to 8-fold higher level of resistance compared with that of a susceptible population (Culpepper et al. 2006). In some cases, the gene amplification of *EPSPS*—normally a low-copy gene in plants (Gaines et al. 2010)—was quite drastic, with resistant plants found to contain from 5-fold to over 160-fold more copies of *EPSPS* than the susceptible plants. This causes increased expression of EPSPS, which is thought to be the mechanism of resistance to glyphosate in this species. To date these are the only three known resistance mechanisms occurring in natural weed populations. Studies are currently in progress to determine the mechanism conferring resistance to glyphosate in waterhemp. In the meantime, weed scientists have become interested in determining whether transferal of glyphosate resistance or other types of herbicide resistance may be expected to occur between waterhemp and other weed species.

1.5 Evolution of Herbicide Resistance through Hybridization

It is apparent that waterhemp is adept at quickly evolving resistance to herbicides. This is due to the interaction of the high level of genetic diversity in this species with the intense selection pressure applied on this species by herbicides. As noted earlier however, this genetic diversity need not all come from within the species itself. Hybridization with other closely-related *Amaranthus* spp. may also provide the genetic variation required for the evolution of herbicide-resistant biotypes. Several studies have documented the ability of waterhemp to hybridize with other closely-related weed Amaranths—specifically Palmer amaranth and smooth

pigweed—and some of these studies have shown that herbicide resistance traits may be transferred across species.

In 1999, Wetzel et al. reported that ALS-resistance could be successfully transferred from Palmer amaranth to waterhemp through hybridization, and this was confirmed in a study conducted by Franssen et al. (2001). In a similar study conducted in 2007, Trucco et al. reported very low proportions of hybrid progeny from such crosses, suggesting that while such transferal of resistance traits between these species is possible, it may be a relatively rare occurrence.

In 2002, Tranel et al. reported successful transferal of ALS-resistance from smooth pigweed to waterhemp in the greenhouse through hybridization and two subsequent backcrosses to waterhemp. Further studies conducted by Trucco et al. (2005a, 2005b) demonstrated that these species hybridize frequently under field conditions. Thus, it may be expected that other types of resistance traits could potentially be transferred through similar hybridization events. Of considerable interest currently is whether glyphosate-resistance may be transferred from waterhemp to other closely-related *Amaranthus* species, and to smooth pigweed in particular, as this would mark yet another blow to the already-decreasing utility of glyphosate as a cure-all in chemical weed control.

1.6 Research Objectives

Waterhemp is a prolific seed producer, capable of producing on the order of 1×10^6 seeds per female plant under ideal growing conditions (Steckel et al. 2003). These seeds are persistent in the soil, with 11% remaining viable for 4 years and 1–3% capable of germination after 17 years. Therefore, an important aspect of a weed management program for waterhemp is the prevention of seed production, for which knowledge of seed production biology is helpful. The

amount of time required for this species to produce viable seeds after pollination, as well as the level of seed dormancy versus the amount of time the seed remains on the female plant is investigated in Chapter 2.

Although glyphosate-resistant waterhemp was first identified in Missouri in 2005, the mechanism of resistance is still unknown. The inheritance and level of dominance of this trait could have implications for both treatment and prevention of the spread of glyphosate resistance in this species. Knowledge of the inheritance of glyphosate resistance could also aid in predicting the rate at which this trait can be expected to spread. Chapter 3 addresses studies conducted to determine the level of dominance of glyphosate resistance, the number of genes responsible for conferring this trait, as well as the resistance mechanism in the Missouri population.

The studies on the genetics of glyphosate resistance in the Missouri population discussed in Chapter 3 involved the crossing of glyphosate-resistant plants with individuals of a population shown to be resistant to PS II inhibitors, ALS inhibitors, and PPO inhibitors. In Chapter 4, F_2 lines created from these initial crosses are screened for the presence of all four resistance types, and potential genetic linkage among herbicide resistance traits is investigated.

Since the evolution of glyphosate resistance in the Missouri populations, there have been reports of waterhemp populations in Illinois that have not been well controlled by glyphosate. Failure to control these populations with treatments of other herbicides has raised a question as to whether these populations may contain multiple resistance, and this possibility is investigated along with the level of glyphosate-resistance in these populations in Chapter 5.

As waterhemp and smooth pigweed have been reported to be among the most prevalent weeds in Illinois, these species can be frequently found growing near one another. Furthermore, waterhemp and smooth pigweed have been demonstrated to frequently hybridize with one

another in the field. Although reports of glyphosate resistance in waterhemp are becoming more common, this resistance has not yet been observed in smooth pigweed. Therefore, it seems reasonable to question whether glyphosate resistance can be successfully transferred from waterhemp into smooth pigweed via hybridization, and this question is investigated in Chapter 6.

1.7 Attributions

Much of the material presented in Chapter 2 was previously published in *Weed Science* 2010, issue 58, pages 167–173 under the title "Time Requirement from Pollination to Seed Maturity in Waterhemp (*Amaranthus tuberculatus*)" by Michael S. Bell and Patrick J. Tranel.

In Chapter 3, Chance Riggins performed all of the work in optimizing the quantitative real-time PCR (qPCR) for use with waterhemp. He also made valuable contributions in writing the qPCR optimization section in the Materials and Methods, and he helped in analysis of some of the results of qPCR experiments.

Chance Riggins provided qPCR data for Brown County plants in Chapter 5. I grew the plants, screened them with glyphosate, recorded visual ratings and dry weights, and extracted and diluted the DNA for the qPCR reactions performed on the Brown County plants. Chance Riggins also suggested primers to use for amplification of *EPSPS* for the sequencing of this gene in the Brown County population—these were primers that had been designed by Gaines et al. (2010) for use in qPCR performed on Palmer amaranth, which happened to amplify the region of the gene needed for sequencing.

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CHAPTER 2

SEED MATURATION TIME IN WATERHEMP

2.1 Abstract

Experiments were conducted to determine the amount of time required for waterhemp to produce mature seeds after pollination. A waterhemp population designated as ACR was used due to its low level of seed dormancy. Female waterhemp plants were pollinated over a 24-h time period and then isolated from males. Two branches, each containing at least 500 flowers, were harvested from each female at the time of the initial pollination, designated as 0 d after pollination (DAP), as well as at multiple other times after pollination up to 62 DAP. One branch from each harvest was stored at 30 C for 48 hrs, while the other branch was stored at -20 C for 48 hrs. Branches were then stored at room temperature until all harvests were complete, at which time seeds from each branch at each time after pollination were collected, weighed and stratified. Germination tests were then conducted to determine the time at which seeds become viable after pollination. Seeds that had not germinated by the end of the germination tests were subjected to tetrazolium testing for viability. Germination tests were also conducted on non-stratified seeds to investigate changes in seed dormancy that were expected to occur over the amount of time the seeds were allowed to remain on the plants. Seeds stored initially at 30 C postharvest became viable 7 to 9 DAP, while seeds stored initially at -20 C postharvest did not become mature until 11 DAP. Seed coat color was white soon after pollination and became dark brown to nearly black by 12 DAP, and seed weight increased until 12 DAP. Tetrazolium tests for seed viability correlated well with the germination tests. Germination tests on non-stratified seeds indicated that dormancy level was initially high in the population used, but began to decrease between 15

and 30 DAP. Results of this study have implications both for waterhemp management and research.

2.2 Introduction

Waterhemp is a dioecious summer annual weed indigenous to the midwestern United States. It is a C_4 plant with a rapid growth rate, which can produce many seeds under ideal growing conditions. However, it has only recently become a prevalent weed throughout much of the midwestern corn (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) production areas (Hager et al. 1997). Waterhemp has become a major problem weed for several reasons. First of all, it is a small-seeded broadleaf. Thus this weed has found its niche in agronomic cropping systems with the widespread adoption of no-tillage practices. A lack of tillage allows the seeds to remain on the soil surface where they can easily grow after germination, whereas burial of waterhemp seeds by tillage can greatly reduce germination and emergence (Hager et al. 1997). Another factor contributing to the weediness of this species is that its seeds germinate throughout much of the growing season, making season-long control with a single herbicide application difficult (Hager et al. 1997).

The multiple herbicide applications required for season-long control of waterhemp have facilitated the appearance of yet another characteristic that contributes to the weediness of this species—the rapid evolution of herbicide resistance (Jasieniuk et al. 1996). In fact, waterhemp has evolved resistance to herbicides with four different modes of action—namely triazines (Anderson et al. 1996), acetolactate synthase- (ALS-) inhibitors (Horak and Peterson 1995), protoporphyrinogen oxidase-inhibitors (Shoup et al. 2003), and most recently glyphosate (Legleiter and Bradley 2008). All four of these resistances may be nuclear inherited and thus can

be spread through pollen-flow (Bell et al. unpublished data; Patzoldt et al. 2003; Shoup et al. 2008; Tranel and Wright 2002). Waterhemp is a prolific species, as a single female plant may produce up to 1×10^6 seeds (Steckel et al. 2003). Consequently, if the ovules on a female plant are fertilized by pollen containing herbicide resistance alleles and those ovules develop into mature seeds, eradication of herbicide-resistant waterhemp plants from a field will become very difficult. Thus, an important focus of any weed management strategy involving waterhemp control should be the prevention of seed production, which entails having some knowledge of the seed maturation biology of this species.

The primary objective of this study was to determine the amount of time required for female waterhemp plants to produce viable seed after being pollinated. Secondary objectives included determination of the length of the seed-filling period based on seed weight, as well as an attempt to determine whether the seeds become dormant during or after the maturation process. To address these objectives, the basic strategy was to fertilize ovules during a controlled pollination event and then to subsequently harvest flowers from the female plants at select times after the controlled pollination event. For the purposes of this paper, a mature seed is defined as a seed that is viable. Maturation will be used to refer to the set of processes that occur from the time of pollination until a seed becomes viable. And finally, dormancy will be defined as the failure of a mature seed to germinate under ideal conditions.

2.3 Materials and Methods

2.3.1 Preparation of plants

The waterhemp population used in this study, designated as ACR, was described previously (Patzoldt et al. 2005). This population was chosen because it tends to have a lower

level of dormancy than most of the other populations that were immediately available for use in this study—an important aspect in an experiment in which seed viability is determined by performing germination tests.

Multiple females were required to perform this experiment. However, plants could not be identified as females until flowering began. Thus, 20 plants were grown for this study with the hope that at least 5 of them would be female. Seeds were sown in a 12 cm x 12 cm x 5 cm container in a medium consisting of a 3:1:1:1 mixture of commercial potting mix[1] to soil to peat to sand. When the seedlings exhibited two true leaves, they were transplanted into 7.6 L pots containing commercial potting mix[1]. Plants were fertilized as needed using a slow-release complete fertilizer[2], and the plants were grown in the greenhouse under mercury halide and sodium vapor lamps that provided a minimum photon flux of 800 μmol m^{-2} s^{-1} at the plant canopy in addition to the light incident from the sun. The lamps were programmed for a 16-h photoperiod, and the greenhouse was maintained at temperatures of 22 C at night and 28 C during the day.

In addition to multiple females being required, numerous flower branches from each of the females were required to allow for germination tests at multiple times after pollination. Therefore, when the plants reached approximately 40 cm in height, the apical meristems were removed. This released the axillary buds from apical dominance, allowing these buds to produce new growth, which ensured that numerous branches were present on each plant at the onset of flowering.

Besides the requirement that each plant contain multiple branches, numerous non-pollinated flowers were needed on each of these branches in order to perform the germination tests. To ensure that each branch contained a sufficient number of non-pollinated flowers, female

plants were isolated from the males by moving them to a separate greenhouse room as soon as they were identified as females. Once isolated, the female plants were allowed to continue to grow in the greenhouse until abundant stigmas were visible on each branch, at which time the plants were pollinated.

2.3.2 Pollination

On the morning of pollination, the females were reintroduced to the room containing the male plants. Male plants were then selected one at a time, held above the female plants and shaken to release pollen. This was done in the morning, as that is the time at which many species, including some in Amaranthaceae, produce the most pollen (Rodríguez et al. 2000; Singh and Babu 1980). We assumed that the same would hold true for waterhemp, although Singh and Babu (1980) also suggest that some species in Amaranthaceae may actually produce the majority of their pollen in the afternoon.

After each of the male plants was used to pollinate the females, the female plants were kept among the males for 24 h after which the males were once again used to pollinate the female plants. Immediately following the second pollination, the females were again isolated from the males in order to prevent any later uncontrolled pollination from occurring. Also at this time the harvest phase of the experiment began.

Included as a test to check for apomixis and uncontrolled pollination were two additional female plants. These plants also were isolated from the males as soon as they were identified as female plants. However, these additional two females were never reintroduced to the room containing the male waterhemp plants. Thus, any seed collected from these two females resulted from one of the following: apomixis, uncontrolled pollination that occurred prior to isolation of

the females, or uncontrolled pollination due to the presence of foreign pollen in the female isolation room.

2.3.3 Harvests

In the first run of the experiment, branches were harvested from six female plants at 0, 3, 5, 7, 9, 12, 14, and 16 d after pollination (DAP). In the second run of the experiment, branches were harvested from five female plants at 0, 3, 5, 7, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 24, 30, and 62 DAP. The harvests consisted of collecting two branches, each containing an estimated number of at least 500 flowers, from each female at each harvest time. One of each pair of branches was incubated 48 h at -20 C while the other was incubated 48 h at 30 C. These two postharvest treatments were chosen as different attempts to achieve our goal of abruptly stopping seed maturation without damaging seeds that may not be fully developed (and therefore fragile). Further, these two postharvest treatments simulated, respectively, a frost event or the fate of seed in a harvested field. After the initial treatment at -20 C or 30 C, branches were stored at room temperature until all harvests were completed, at which time seeds were prepared for germination testing.

2.3.4 Germination tests

Seeds were manually harvested from each branch. A digital camera[3] connected to a microscope[4] was used along with computer software[5] to photograph a random assortment of seeds from each branch. In each random assortment of seeds, a range of maturity levels was observed from infancy up to a maturity level that the majority of the observed seeds had reached, designated as the most prevalent maturity level (MPML). Next, seeds at the MPML from each

treatment (ten seeds/rep in the first run, and twenty seeds/rep in the second run) were randomly selected. One such seed from each treatment and rep was photographed in order to record seed color and to compare sizes and shapes of seeds at different times after pollination. Each of the seeds were then surface-sterilized by soaking for 10 min in a 1:1 bleach:water solution. The seeds were then stratified in sterile deionized water at 4 C for at least 10 d, after which time they were placed on moist filter paper in petri dishes. The petri dishes were kept in an incubator at temperatures of 35 C during the day and 30 C at night, as it has previously been shown that alternating temperatures improve waterhemp seed germination (Leon et al. 2006; Steckel et al. 2004). After one week, the fraction of seeds germinating out of the total number of seeds initially present in each plate was recorded and a germination percentage calculated.

For the second run of the experiment, 20-seed samples were weighed prior to stratification. Additionally, extra 20-seed samples were collected from the branches harvested at 10, 15, 30, and 62 DAP, which had been subjected to the 30 C postharvest treatment. Germination tests were conducted on these samples without stratification to investigate possible changes in the level of dormancy over time in this population.

2.3.5 Mold-growth tests

Based on preliminary work involving germination of seeds on filter paper in petri dishes, it was decided to conduct a small experiment on methods of preventing mold growth on the filter paper during germination tests. Before beginning the experiment, filter paper and petri dishes were subjected to germicidal ultraviolet light in a sterile fume hood for 20 min, with filter paper receiving a 10 min treatment on each side. Germination tests were then conducted on ACR seeds (which had been sterilized and stratified as described above) in the petri dishes, with nine

treatments being applied, each consisting of three experimental replicates. Five of these nine treatments consisted of seeds being placed on filter paper, which was moistened as needed with sodium hypochlorite (bleach) solutions at concentrations of 916, 4580, 9160, 45,800, and 91,600 µM. Bleach solutions were made by diluting bleach stock[6] with sterile deionized water. Two of the remaining four treatments consisted of filter paper being kept moist with sterile deionized water, with seeds being placed on the filter paper for one treatment, and no seeds used in the second treatment. The remaining two treatments consisted of filter paper first being soaked in 100% ethanol, which was then allowed to dry. The filter paper was then placed in petri dishes and kept moist with sterile deionized water. In one of these treatments, seeds were added to the paper, while in the other treatment no seeds were used. During the course of this experiment, petri dishes were only opened inside a sterile fume hood. For the remaining time in the experiment, petri dishes were kept in an incubator at 30 C. Plates were observed twice per day for 14 days to ensure that the filter paper remained moist.

2.3.6 Tetrazolium tests

After the seven d germination test in the second run of the experiment, the viability of the stratified seeds that had not yet germinated was examined using a tetrazolium (TZ) test. Harvest times investigated were 7, 10, 13, and 30 DAP, for both of the postharvest treatments. Ungerminated seeds from each of the female plants were pooled for each of these eight treatments, and six seeds were then randomly selected from each treatment for TZ testing. To perform the TZ tests, the seeds were placed in a line on damp filter paper in a petri dish. The seeds were held with forceps while they were cut longitudinally with a scalpel to expose the embryos. After being sliced, the seeds were placed face down on the filter paper, keeping the 2

halves of each seed in close proximity to one another. Approximately 1 ml of 1% TZ (w/v) solution was applied to the filter paper in each petri dish, after which the dishes were covered and placed in the dark for 24 h. After 24 h of exposure to TZ, the seed-halves were turned over and investigated for embryo staining. Due to the small size of these seeds and the ease with which such small embryos can be damaged during the slicing process, seeds were scored as viable if any purple staining was apparent on an intact embryo or an embryo fragment. The overall procedure was modeled after that of Peters (2002).

In addition to testing the ungerminated seeds remaining in the petri dishes after the germination tests, 20-seed samples from each of the five 13 DAP/30 C postharvest replicates were combined to make a 100-seed pool. The same was done separately from the 13 DAP harvests that had received the -20 C postharvest treatment. Ten seeds were randomly selected from each of the 100-seed pools and subjected to TZ testing, as was described above.

2.4 Results and Discussion

2.4.1 Mold-growth

Observations recorded after the first 48 hours of this experiment showed no indication of mold growth on any of the plates. After 14 days, only one plate contained mold, which was one of the three replicate plates containing seeds on filter paper that had originally been soaked in ethanol. This is an interesting result, but the cause of this effect was not investigated. That it only occurred in one of the three plates receiving this treatment can perhaps be attributed to random experimental error. The observation of mold on only one out of 27 plates was much different than the observations made in preliminary germination tests, in which mold was observed on over 30% of the plates used in the study. One major difference between the germination tests in

the preliminary studies and those in this study on mold growth was that plates in the mold growth experiment were only opened inside a sterile fume hood, while in previous studies plates had been opened in the lab, which was apparently the main source of mold spores.

Another interesting finding was that seed germination was totally inhibited on filter paper moistened with bleach at concentrations of 45,800 µM and higher. Germination did occur for all lower-concentration bleach treatments, although the radicals stopped growing shortly after emergence from the seed coat on the filter paper moistened with 9,160 µM bleach. Also, rather unsurprisingly, the seed coats changed color under these two highest-concentration bleach treatments from nearly black to a light brown color. Based on the results of this test, it was decided that sterile deionized water would be used to moisten the filter paper in subsequent germination tests, and that the plates would only be opened when inside of the sterile fume hood.

2.4.2 Seed collection

For several of the harvests, including some that were collected as early as 0 DAP, mature-looking seeds were found. However, this amounted to a maximum of two seeds in a particular treatment, or less than one percent of all the seeds or flowers observed in each treatment. These mature-looking seeds were dark brown or nearly black in color. The presence of these seeds at such early harvest times is likely due to pollination that occurred before the plants were identified as females and subsequently isolated from the male plants. However, as the frequency of these mature-looking seeds was extremely low—amounting to less than 1% of all of the seeds analyzed from a particular treatment—such seeds were ignored. What remained then was a large number of seeds that all appeared to be at the same maturity level, designated as the most prevalent maturity level (MPML). Reference to the MPML becomes important when

investigating seeds at later harvest times, as at nearly every harvest time a range of seed maturity levels were represented from infancy up to the MPML of the given harvest time. This is likely due to embryo abortion in the pollinated plants, as the non-pollinated females produced very few seed even at the later harvest times.

Although mature-looking seeds were also collected from the non-pollinated plants at all harvest times, these seeds were present in fewer than one percent of the flowers observed, and thus any possible effects of early pollination, foreign pollen entering the female isolation room, or apomixis were considered insignificant sources of error in this study.

2.4.3 Seed appearance

At 0 DAP nothing was found that bore any resemblance to a seed after observation of approximately 500 flowers per treatment. In fact, nearly every flower appeared empty at this harvest time. By 3 DAP ovules were visible (Figure 2.1). They appeared as thin, translucent discs, which were light tan in color. Some darkening occurred over the next two d, and by 5 DAP the seeds receiving the 30 C postharvest treatment (oven-treated) were a matte tan-brown color, while those seeds receiving the -20 C postharvest treatment (cold-treated) were a glossy light tan color. Both oven- and cold-treated seeds were still relatively thin at this point. By 7 DAP, seeds had continued to darken, reaching a reddish-brown color for both oven- and cold-treatments. A notable difference between these two treatments at this stage was that the cold-treated seeds were rounded, appearing to already be nearly filled, while the oven-treated seeds still appeared to be relatively thin. Seeds from both postharvest treatments displayed glossy seed coats by this time. At 9 DAP, seed color had darkened even more, and by 12 DAP, all seeds appeared to finish darkening, reaching a terminal color of dark brown to black when viewed under a dissecting

microscope. As for visual filling, the cold-treated seeds appeared nearly full by 7 DAP, and totally full by 14 DAP, while the oven-treated seeds showed a more gradual increase in width that also appeared to terminate at approximately 14 DAP.

2.4.4 Seed weight

Analysis of the 20-seed weights painted a slightly different picture of the seed filling process when compared with the visual observations, as the weights of both the oven-treated and the cold-treated seeds increased at the same rate when compared at multiple times after pollination (Figure 2.2). Seed weight appeared to increase approximately linearly with time after pollination between 7 DAP and 12 DAP, at which time the seeds attained their maximum weight. The 20-seed weights remained constant from 12 DAP until the conclusion of the experiment at 62 DAP. The fact that the same pattern of weight increase occurred for both postharvest treatments is rather interesting as the cold-treated seeds appeared fuller than the oven-treated seeds as early as 7 DAP when viewed under a dissecting microscope. Why these cold-treated seeds appeared fuller than the oven-treated seeds while sharing the same weight is an interesting question that was not addressed in this study.

2.4.5 Germination tests

In the first run of the experiment, six females were pollinated using ten males, and samples of ten seeds at the MPML were collected from each harvest. The one or two mature-looking seeds present at some of the earlier harvest times were included in these germination tests, which explains the apparent non-zero germination percentages at 5 and 7 DAP (Figure 2.3 A). In fact, the only germination that occurred from these harvest times was due to those seeds

that already displayed a dark brown or purple color. However, no seeds at the MPML began germination until 9 DAP, at which time germination of the oven-treated seeds reached approximately 12%. Germination of the oven-treated seeds reached a maximum of approximately 78% by 12 DAP, after which point it leveled off and remained at or near 75% for the remainder of the harvests collected.

The germination profile of the cold-treated seeds was slightly different, in that germination did not begin until 12 DAP, at which time it reached about 25%. By 14 DAP the germination percentage reached a maximum of 49%, after which it dropped slightly to 31% by 16 DAP. Based on the results of this run, we designed the second run of the experiment to provide more germination data between 9 and 12 DAP, as well as to provide more data at later time points. In addition, we chose to perform the germination tests only on seeds at least as old as 7 DAP, as it was assumed that once again no germination would occur in the MPML seeds prior to that time point.

Analysis of the second run, in which 6 females were pollinated using 8 males, shows a similar trend to what was previously described for the first run. The oven-treated seeds did not begin to germinate until 9 DAP, at which time approximately 32% of those seeds at the MPML demonstrated viability (Figure 2.3 B). Germination of these oven-treated seeds was maximized by allowing one more d of on-plant maturation, as germination reached about 79% by 10 DAP and 11 DAP, after which point the percentage dropped slightly, hovering near 70% until 24 DAP when it again approached 80%.

For the cold-treated seeds, germination began by 10 DAP with about 5% of the seeds at the MPML germinating. By 11 DAP that percentage had increased to 13%, and at 12 DAP it reached about 40%, where it remained until 15 DAP. The percentage of cold-treated seeds

germinating again increased at 16 DAP to over 50%, after which point it continued to gradually increase to eventually match that of the oven-treated seeds near 80% for the 24, 30, and 62 DAP harvest times.

The trends obvious from these data are that the seeds stored at 30 C postharvest for 48 h became viable at a time between 7 and 9 DAP, which was at least one d earlier than the cold-treated seeds became viable. It is also interesting to note that by 12 DAP the oven-treated seeds reached their maximum germination percentage near 80%, while the cold-treated seeds approached this apparent limit to germination more gradually, only reaching this level by approximately 3 weeks after pollination. This could suggest either that cold temperatures caused damage to the seeds that prevented them from germinating, or that the oven-treated seeds continued to mature for some time as the plant material dried after the harvest had been made. However, based on the fact that nearly 80% germination was attained by oven-treated seeds by just 12 DAP, while cold-treated seeds required approximately twice that time to attain the same germination percentage, it seems more likely that freezing caused some damage to the otherwise viable seeds until about 24 DAP, after which time they became immune to freezing damage.

2.4.6 Tetrazolium tests

None of the ungerminated seeds analyzed from the 7 DAP harvests showed any signs of staining for either of the postharvest treatments (data not shown). This was expected because none of the seeds harvested 7 DAP germinated. By 10 DAP, only 17% of the cold-treated seeds were viable, while 83% of the oven-treated seeds were viable. This is in fairly close agreement with the viability estimates that may be inferred from the germination data in Figure 2.3. Analysis of ungerminated seeds harvested 13 DAP showed that cold-treated and oven-treated

seed were 83% and 100% viable, respectively. By 30 DAP, 100% of the seed were viable, indicating the on-plant maturation was complete. As for the extra 10-seed samples collected by pooling seeds that had not been subjected to germination testing from each of the 5 females at 13 DAP, the oven-treated seeds showed 100% staining, while the cold-treated seeds showed only 60% staining—still in close agreement with the germination data in Figure 2.3.

2.4.7 Dormancy

Results of the dormancy test, in which oven-treated seeds were tested for germination without stratification, revealed an intriguing pattern of dormancy versus time after pollination. The seeds are apparently dormant soon after fertilization occurs, as very few of the seeds harvested at 10 DAP and at 15 DAP germinated after seven d of germination testing (Figure 2.4). Interestingly, by 30 DAP, approximately 35% of the tested seeds germinated, and the percentage of seeds germinating from the 62 DAP harvests was nearly identical to that of the 30 DAP harvests. It has been shown that these oven-treated seeds are indeed mature by 10 DAP (Figure 2.3). In fact, nearly 80% of oven-treated seeds harvested at 10 DAP germinated after stratification, and of those that did not germinate during germination testing, 83% were viable based on TZ testing. Thus, the fact that none of these seeds germinated under ideal conditions without being stratified indicates a high level of dormancy in the young seeds. Apparently the dormancy level began to decrease at some point between 15 and 30 DAP. Perhaps this could be beneficial to the species, because if young seeds had low levels of dormancy they would germinate in the fall and would probably be killed by frost before producing seeds. It should be noted, however, that this trend of dormancy versus time may be biotype-specific. In fact, differing levels of dormancy have previously been reported for three other waterhemp

populations by Leon et al. (2006), in which the authors suggest that seed dormancy is an adaptive trait that may be influenced by agricultural practices.

The data from this study suggest that waterhemp seeds may become mature as soon as 9 DAP. Thus, once pollen begins to spread in a field containing female waterhemp plants, a very narrow window of time exists in which a farmer can work to prevent seed production. Seed maturation biology has been studied in many other species, including some important weeds in agronomic cropping systems, and the results show that the time required for seed maturation after pollination varies among species. For instance, Egley (1976) reported that prickly sida (*Sida spinosa* L.) seeds are incapable of germination until 12 d after anthesis, and that the seeds attain their maximum dry weight by 14 d after anthesis. Prickly sida seeds are capable of 80% germination at 12 to 16 d after anthesis, after which time germination drops to nearly 0%, presumably due to the onset of dormancy (Egley 1976). Chandler et al. (1977) reported that in purple moonflower (*Ipomoea turbinata* Lag.), seeds first acquire the ability to germinate at 20 d after anthesis. They also reported that maximum germination occurs at 26 d after anthesis, and that maximum dry weight occurs at 34 d after anthesis. Similarly, Jayasuriya et al. (2007) reported that in pitted morningglory (*Ipomoea lacunosa* L.), germination does not occur until at least 20 DAP. These seeds reach their maximum dry mass by 22 DAP and their maximum germination percentage at 24 DAP, but by 30 DAP the seeds no longer germinate unless manually scarified, indicating that dormancy is due to impermeable seed coats (Jayasuriya et al. 2007). Even in arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), a species known for its short life cycle, Koorneef et al. (1989) found that seeds do not become viable until at least 13 DAP when dried immediately after harvest. Thus, our results indicate that waterhemp seeds become mature in as little time or less than that required for seeds of these other species.

One implication of the results of this study is directly applicable to scientists crossing waterhemp plants to perform progeny analysis or for bulk seed production. For rapid generation advancement in this species, one apparently need not wait more than 14 DAP to collect seeds from female plants. In fact, it is possible to collect many mature seeds by as early as 10 DAP if these seeds are dried immediately after harvest and then stratified for at least 10 d. Thus, potentially in as little as 20 d after flowering begins, one may begin growing the next generation of waterhemp plants. For long-term seed storage, however, as may be desirable in situations where the objective of the cross is simply to increase seed supply, it may be advisable to wait until at least 20 DAP before collecting seeds. Our results suggest that by this time the seeds may be safely stored at low temperatures without any obvious negative effects on viability.

From this study we conclude that waterhemp seeds become mature in less than two weeks after pollination has occurred. Seed color darkens and seed weight increases linearly until approximately 12 DAP. Finally, waterhemp seeds exhibit high levels of dormancy at an early age, and these dormancy levels begin to drop between 15 and 30 DAP. More research is needed to determine whether environmental or biotypic effects significantly impact seed maturation time in waterhemp.

2.5 Sources of Materials

[1] LC1 Professional Growing Mix. Sun Gro Horticulture Canada Ltd. 52130 RR 65, P.O. Box 189, Seba Beach, AB 70E 2BO Canada. Distributed by Sun Gro Horticulture Distribution Inc. 15831 N.E. 8th St., Suite 100, Bellevue, WA USA 98008.

[2] Scotts Osmocote Classic 13-13-13 Slow-Release Fertilizer. The Scotts Company LLC, 14111 Scottslawn R., Marysville, OH 43041.

[3] SPOT Insight QE Color Model 4.2.1. Diagnostic Instruments, Inc. 6540 Burroughs St., Sterling Heights, MI 48314.

[4] Nikon SMZ800 Stereoscopic Zoom Microscope. Nikon Inc., 1300 Walt Whitman Rd., Melville, NY 11747-3064 USA.

[5] SPOT Imaging Software, version 3.5.0 2002. Diagnostic Instruments, Inc. 6540 Burroughs St., Sterling Heights, MI 48314.

[6] Clorox Ultra® Bleach. 6.15% sodium hypochlorite (w/w). The Clorox Company, 1221 Broadway, Oakland, CA 94612.

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2.8 Figures

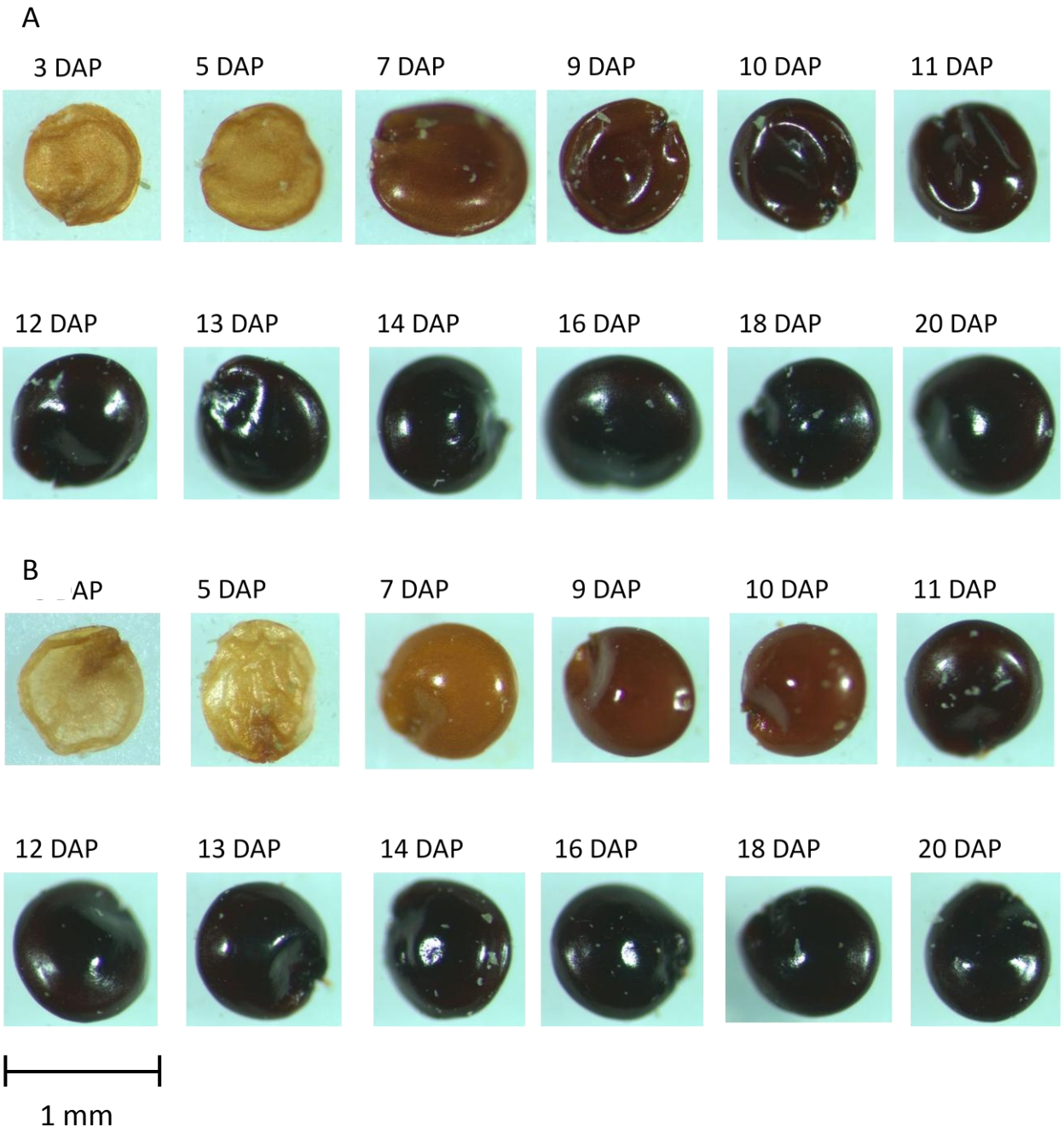

Figure 2.1 Pictures of seeds collected at the most prevalent maturity levels at selected d after pollination (DAP) receiving either a 48-h postharvest treatment of 30 C (A) or a 48-h postharvest treatment of -20 C (B).

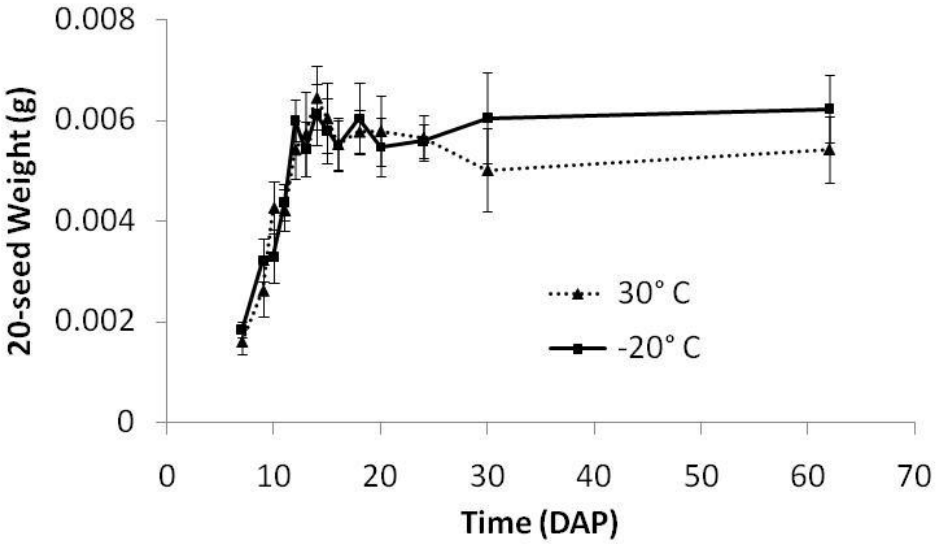

Figure 2.2 Mean 20-seed weight versus d after pollination (DAP) for seeds receiving a 48-h postharvest treatment of storage at 30 C (▲) or -20 C (■). Vertical bars represent ± SEM (n = 5).

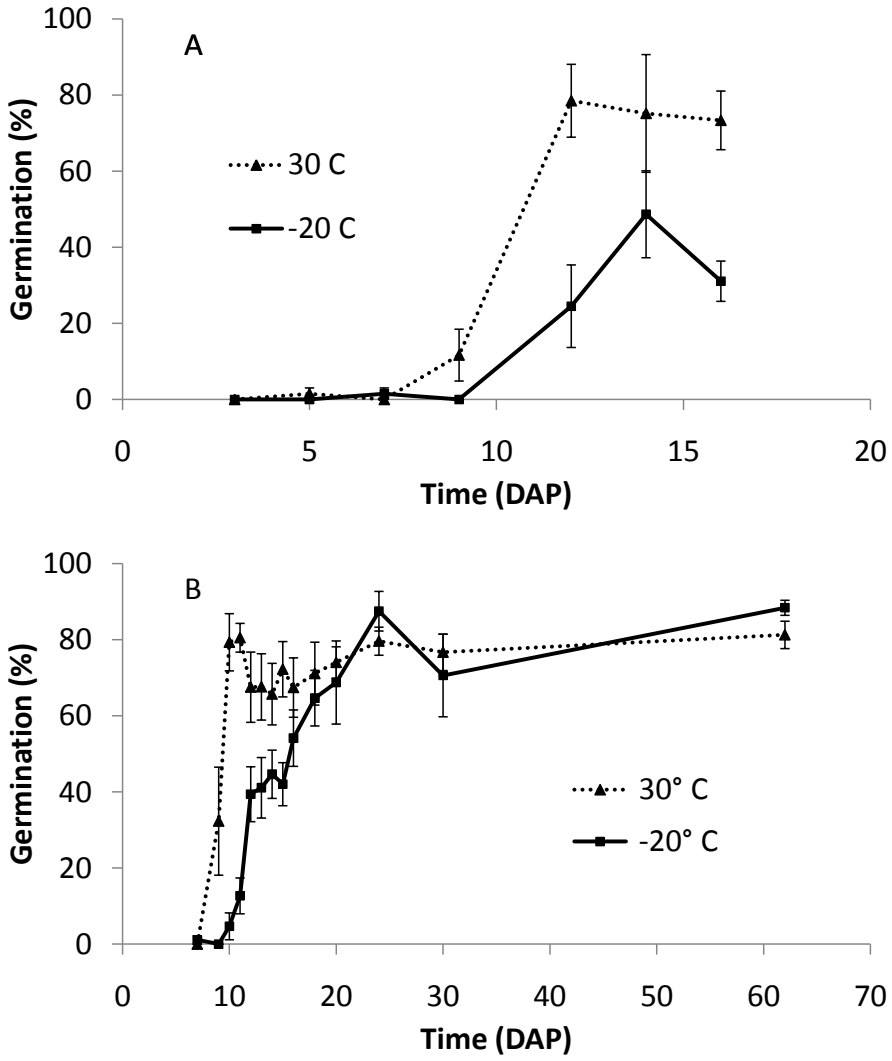

Figure 2.3 A. Mean germination percentage of seed samples versus d after pollination (DAP) from the first run of the experiment receiving a postharvest treatment of 30 C for 48 h (▲) or -20 C for 48 h (■). Seeds were first stratified for 10 d, then incubated at 35 C during the day and at 30 C at night for 7 d during the germination testing. B. Mean germination percentage of seed samples from the second run of the experiment receiving a postharvest treatment of 30 C for 48 h (▲) or -20 C for 48 h (■) versus d after pollination (DAP). Seeds were first stratified for 30 d, then incubated at 35 C during the day and at 30 C at night for 7 d during the germination testing. Vertical bars in both panels represent ± SEM (n = 6 in A, n = 5 in B).

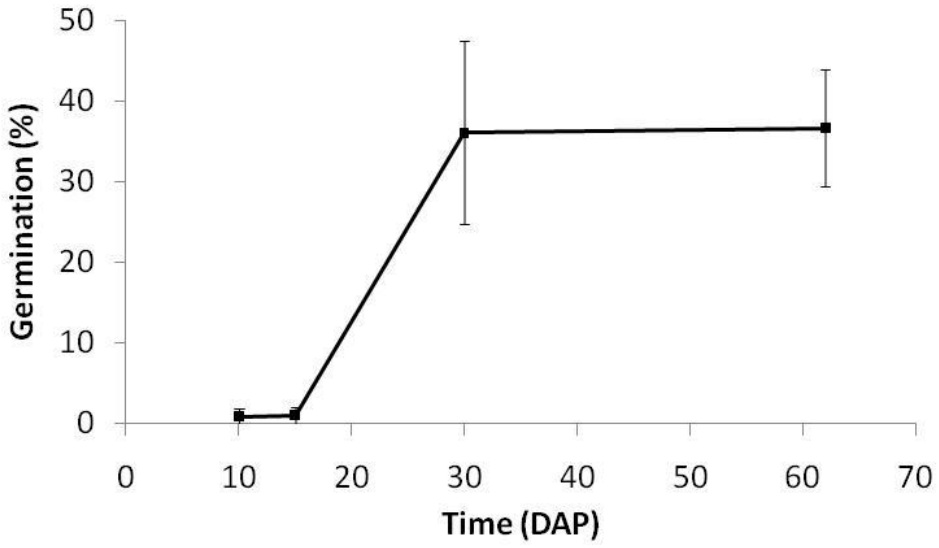

Figure 2.4 Mean germination percentage of non-stratified seeds receiving a postharvest treatment of 30 C for 48 h versus d after pollination (DAP). Vertical bars represent ± SEM (n = 5).

CHAPTER 3

GENETICS OF GLYPHOSATE RESISTANCE IN A MISSOURI WATERHEMP POPULATION

3.1 Abstract

A Missouri waterhemp population (MO1) was investigated for inheritance and potential mechanism of glyphosate resistance. MO1 plants were screened with glyphosate to identify resistant (R) individuals, which were then crossed with plants from a susceptible population (ACR) to created F_1 lines. Crosses were performed in both directions, to determine whether glyphosate resistance was nuclear-inherited or maternally inherited. An additional objective was to create a homozygous resistant waterhemp line through the utilization of clones, in which F_1 lines were also created. F_1 plants were screened with several rates of glyphosate and evaluated for resistance level. All F_1 lines showed segregation for resistance to some degree. The level of resistance appeared to be slightly lower than that of the MO1 population, which may indicate that glyphosate resistance is a partially dominant trait. Resistant individuals were identified from both directions of crosses, indicating that glyphosate resistance is nuclear-inherited. Despite the segregation in the F_1 lines, resistant individuals were selected from one line that demonstrated a lower level of segregation than two of the other lines tested, and these individuals were used to create F_2 and backcrossed lines (BC_S), where backcrosses were performed by crossing back to a susceptible parent. Progeny of these lines were then screened for resistance to glyphosate at rates of 1680 and 3360 g ae ha^{-1} and segregation ratios were analyzed. The expected segregation ratio in the BC_S lines was 0:1:1 (R: intermediate (I): S). The observed ratios failed a chi-square (χ^2) test for a single gene trait based on the expected segregation, giving p-values of < 0.0001 for

both doses tested. However, at both doses, some F_1 plants expected to show an intermediate phenotype were killed by the herbicide, and the expected segregation ratios were modified accordingly. In this case the BC_S lines at the lower dose passed the single-gene χ^2 test with a p-value of 0.273, although they still failed to pass at the higher rate, with a p-value of 0.025. To avoid problems with differentiating between I and S phenotypes in the F_2s, plants displaying either of these phenotypes were placed into a larger phenotypic class (called S). The expected segregation ratios for a single gene trait became 1:3, and all three of the F_2 lines tested at the lowest rate of glyphosate passed the single gene χ^2 test. However, only one line out of three passed at the higher rate. F_1 lines created using clones also demonstrated a high level of segregation inconsistent with resistance being a single gene trait. Due to these unexpected results the experiment was repeated. During repetition, it was discovered that glyphosate resistance in Palmer amaranth was conferred by gene amplification of *EPSPS*. Waterhemp was tested for *EPSPS* amplification by using quantitative real-time PCR, and MO1 was found to consistently contain 3–5 times the number of copies of the susceptible population. Analysis of F_1s showed that copy number rarely cosegregated with resistance, and that copy number was inherited as a quantitative trait. Analysis of F_2s showed that, again, resistance rarely cosegregated with copy number, although a weak correlation may exist. Inheritance of copy number in F_2 plants appeared similar to that expected for a quantitative trait, which may indicate that extra copies of *EPSPS* are located in different regions of the genome. The weak correlation between resistance level and copy number indicates that another, as of yet unidentified, factor is necessary to confer resistance to glyphosate in waterhemp.

3.2 Introduction

Glyphosate is a broad-spectrum foliar-applied herbicide that was first marketed by Monsanto in 1974. It is the most widely used herbicide in the world (Preston and Wakelin 2008). This is due largely to the commercialization of glyphosate-resistant crops, beginning with Roundup Ready® soybeans marketed by Monsanto in 1996, leading to an increase in glyphosate use from 2.5 million kg yr^{-1} before 1996 to 30 million kg yr^{-1} by 2002 (Young 2006). This increased use has inevitably led to the evolution of herbicide-resistant weed biotypes (Jasieniuk et al. 1996; Heap 2010). The first weed reported resistant to glyphosate was rigid ryegrass [*Lolium rigidum* Gaudin], and to date there are at least 18 other species that have evolved resistance to this herbicide (Heap 2010), including waterhemp (Legleiter and Bradley 2008).

Waterhemp is a small-seeded summer annual plant indigenous to the midwestern United States (Sauer 1955) that has only recently become a major problem weed for corn and soybean producers (Hager et al. 1997). This is thought to be due to several factors, including a recent shift toward no-till practices, allowing the small seeds of waterhemp to remain near the soil surface where they stand a better chance of survival after germination (Buhler 1992). Other factors contributing to the weediness of this species are its high seed production, its prolonged germination period, and its adeptness at evolving resistance to herbicides (Hager et al. 1997). To date, waterhemp has evolved resistance to four different herbicide modes of action (Heap 2010).

The mechanism of glyphosate resistance in waterhemp is unknown. However, a recent report of glyphosate resistance in a related species, Palmer amaranth [*Amaranthus palmeri* S. Wats.] indicated that amplification of the gene encoding for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) was responsible for conferring resistance to this herbicide (Gaines et al. 2010). The authors reported that resistant plants contained as many as 160 times the number of copies of

EPSPS as susceptible plants, which was determined through the use of quantitative real-time polymerase chain reactions (qPCR).

Quantitative real-time PCR has several applications, including gene expression analysis and determination of absolute copy number of a particular gene in a genome (Anonymous 2010). Another useful application is the determination of relative copy number of a particular gene in one plant compared with the copy number of the same gene in another plant, which is the application that was used in the determination of the mechanism of glyphosate resistance in Palmer amaranth (Gaines et al. 2010).

During qPCR, a fragment of a gene is amplified as in standard PCR. The reactions consist of a denaturation phase, in which the temperature is raised to denature double stranded DNA molecules. The temperature is then lowered, allowing primers to anneal to single strands of DNA at a specific position. The temperature is then increased to the optimal working temperature for the polymerase enzyme during the extension phase, in which new copies of the target are synthesized. However, in standard PCR, the results of the reactions are analyzed after completion of the PCR program. In qPCR, on the other hand, products are quantified after each cycle of the reaction with the use of fluorescent dye. Although several dyes exist for use in qPCR, one of the most common is SYBR® Green (Anonymous 2010).

SYBR® Green is an intercalating fluorescent dye, meaning that as single strands of DNA begin to pair as the reaction temperature is lowered after denaturation, the dye molecules bind to the double-stranded DNA molecules, in which state they exhibit a large increase in fluorescence over dye molecules that are not bound to double-stranded DNA (Anonymous 2010). By comparing the amount of fluorescence during a qPCR reaction from one cycle to the next, the amount of increase in double-stranded DNA product produced during the amplification reactions

can be monitored. Starting template concentrations are generally very low, and thus fluorescence cannot be detected in early cycles. However, at some point the amount of product, and thus the amount of fluorescence in the sample, becomes detectable over the background level. The number of cycles required to reach this point for a particular sample is referred to as the threshold cycle (C_t). The value of the threshold cycle is dependent on several factors, such as the amount of starting template. If more template is present at the beginning of a reaction, assuming the concentration of reagents is non-limiting, then such samples should reach the C_t in fewer cycles than samples starting with a lower template concentration. This implies that the results of qPCR, in which C_t values are compared among various samples to make conclusions as to gene expression or copy number, can be very sensitive to initial conditions. Due to the exponential amplification that occurs in PCR, small differences in starting template concentrations can lead to large differences in C_t values. However, there is a way around this potential problem, which involves the use of a reference gene.

The use of a reference gene in qPCR eliminates the critical need for beginning template concentrations to be equal among samples. One of the most important aspects of a reference gene is that it be a single-copy gene. Use of such a gene (for instance acetolactate synthase (ALS) was used as a reference gene by Gaines et al. (2010) and is a single-copy gene well known among weed scientists) removes the need for equal starting concentrations of DNA among samples because for each sample, the C_t value of the reference (single-copy) gene is compared to the C_t value of the target gene. Although the C_t values for both genes depend on the starting concentration of DNA, the difference between the C_t values is independent of the initial conditions, and is useful in providing information solely on the difference in the number of copies of the target gene compared to the reference gene within a particular genome. To compare

copy numbers of a target gene in multiple organisms hypothesized to have an elevated copy number of the gene with the copy number in an organism hypothesized (or known) to have a lower number of copies of the target gene, the quantification process can be further simplified by normalizing the difference in copy number between the target gene and the reference gene of each organism tested to that of an organism known to have a lower copy number of the target gene (and thus a smaller difference between C_t values of the target gene and the reference gene). This is known as the comparative C_t method (Schmittgen and Livak 2008), and it provides a relative copy number between organisms, allowing for the estimation of fold-increases in the number of copies of the target gene in studies on gene amplification, such as that performed on Palmer amaranth (Gaines et al. 2010).

In 2004, two soybean producers in Missouri reported a failure to control waterhemp with glyphosate, and these populations were later confirmed to be glyphosate-resistant (Legleiter and Bradley 2008). The objectives of the current study were to determine the inheritance of glyphosate resistance in this population. Of specific interest were whether the trait was nuclear- or maternally inherited, the level of dominance of the trait, and the number of genes responsible for conferring glyphosate resistance. The starting hypothesis was that glyphosate resistance in this population is conferred by a single, nuclear-inherited, dominant gene.

3.3 Materials and Methods

3.3.1 Plant culture

All plants used in this study were grown from seeds sown in a 12 cm x 12 cm x 5 cm container in a medium consisting of a 3:1:1:1 mixture of commercial potting mix[1] to soil to peat to sand. When seedlings reached the two-leaf stage, they were transplanted into individual 6 cm

x 4 cm x 5 cm inserts in 24 cm x 48 cm x 5 cm flats containing the previously mentioned growth medium. When plants reached 5 cm in height they were transplanted to 12 cm square pots containing 700 ml of growth medium, where they were allowed to grow until completion of the experiment. Plants were fertilized as needed using a slow-release complete fertilizer[2], and the plants were grown in the greenhouse under mercury halide and sodium vapor lamps that provided a minimum photon flux of 800 µmol m^{-2} s^{-1} at the plant canopy in addition to the light incident from the sun. The lamps were programmed for a 16-h photoperiod, and the greenhouse was maintained at temperatures of 22 C at night and 28 C during the day.

3.3.2 Herbicide application

All herbicide applications for this study were made using a compressed air, moving nozzle spray chamber with an adjustable platform and equipped with an 80015EVS even flat spray nozzle[3]. The nozzle was maintained at approximately 45 cm above the plant canopy. The sprayer was calibrated to deliver 187 L ha^{-1} of water at 207 kPa. Plants were returned to the greenhouse immediately after spraying.

3.3.3 Selection of glyphosate-resistant waterhemp

The glyphosate-resistant waterhemp population used in this study was one from Missouri, designated as MO1, which was described previously (Legleiter and Bradley 2008). A waterhemp population from Adams County, Illinois, designated Adams County Resistant (ACR) was used as the susceptible control. This population has also been described previously (Patzoldt et al. 2005). Plants were grown as described above, until they reached 10–15 cm in height, at which time they were treated with herbicide. Glyphosate[4] was applied at rates of 840, 1680, 3360, and 6720 g ae

ha^{-1} and treatments included 2.5% (v/v) ammonium sulfate[5] (AMS). Five ACR and 24 MO1 plants were treated at each herbicide rate. An additional five plants of each biotype were kept as untreated controls. At two weeks after treatment, MO1 plants were rated as resistant (R) or susceptible (S) by comparison of their responses to glyphosate with those of ACR. The multiple herbicide doses were used in order to ensure that at least one of the doses would allow for clear differentiation of R and S plants.

3.3.4 Creation of F_1s

MO1 plants that were confirmed to be resistant to glyphosate were kept in the greenhouse until flowering began, at which time they were used in crosses to create F_1 seeds. Crosses consisted of either a resistant (R) MO1 male or a susceptible (S) ACR male, as well as multiple resistant MO1 females and susceptible ACR females in each cross (Figure 3.1). Crosses were performed either in growth chambers maintained at 28 C during the day and 22 C at night, which were programmed for a 16 hour photoperiod, or in pollination bags[6] in the greenhouse under conditions described above. Male plants were shaken each morning to ensure maximum pollen transfer to the female plants. The crossing was allowed to occur for approximately two months, or until it appeared that the male plant in a particular cross was no longer producing viable pollen. It has been shown that waterhemp may produce viable seed in as little as two weeks after pollination (Bell and Tranel 2010). However, as the female plants had just begun flowering when crosses were established, the crosses were allowed to occur for additional time in the interest of high seed production, by allowing the female plants to continue to produce flowers during the two months of crossing. ACR females were included in crosses with ACR males as a test for

pollen contamination—if glyphosate-resistant progeny were collected from such crosses, it would indicate that foreign pollen had reached the female plant.

When crossing was complete, the female plants were harvested and dried at room temperature for at least two weeks. Seeds were then manually threshed from each plant and stratified. This procedure consisted of surface-sterilizing the seeds for 10 min by soaking them in a 1:1 bleach:water mixture, after which the seeds were rinsed twice with sterile deionized water. The seeds were then suspended in 0.15% (w/v) agarose and stored at 4 C for at least 2 weeks to break seed dormancy.

3.3.5 Creation of clones

An attempt was made to create homozygous glyphosate-resistant waterhemp lines through a technique involving cloning glyphosate-resistant MO1 plants. The plan was to clone multiple female MO1 plants, and to use one such clone from each of the plants in multiple crosses containing either an ACR male or a resistant MO1 male (Figure 3.2). Each cross would also contain at least one ACR female. Progeny from MO1 clone x ACR (R ♀ x S ♂) crosses and from ACR x MO1 (S ♀ x R ♂) crosses would be screened to identify the genotype of the MO1 parent, with the expectation that progeny would segregate either 1:1 R:S if the MO1 parent was heterozygous for glyphosate resistance (Rr) or the progeny would not segregate (i.e., they would all be resistant) if the MO1 parent was homozygous for glyphosate resistance (RR). Assuming successful identification of at least one RR MO1 female clone and one RR MO1 male plant, a homozygous-resistant line could be obtained simply by collecting seed from a clone of the identified RR MO1 female that had been crossed with the identified RR MO1 male plant. The cloning procedure follows.

After confirmation of resistance, apical meristems were removed from approximately 20 MO1 plants to eliminate the effect of apical dominance and thus to promote growth from axillary buds. Such plants were allowed to grow until they contained numerous branches, at which time the plants were cloned.

Clones were made by taking cuttings from resistant plants. In taking the cuttings, a razor blade was used to remove the top 10 cm from branches on MO1 plants, with care taken to cut the stem both at an angle and directly below a leaf, leaving a leaf at the very bottom of the freshly-removed branch (Figure 3.3). This lowest leaf was then cut off at a point at or near the stem of the branch, and this section was coated in rooting hormone[7] and placed in moistened commercial potting mix in a 24 cm x 48 cm x 5 cm flat containing slow-release fertilizer. Eight such cuttings were taken from each resistant plant and the flats containing the cuttings were then placed in a mist room in the greenhouse. The mist room contained no supplemental lighting and was maintained at a temperature of 23 C. Plants were automatically misted with water every 15 minutes to prevent dehydration of plant tissue while awaiting root growth.

Cuttings were kept in the mist room for at least two weeks, or until adequate rooting had taken place, at which time the cuttings were transplanted into 12 cm square pots containing 700 ml of the 3:1:1:1 mixture of commercial potting mix: soil: peat: sand and placed back in the original greenhouse room with supplemental lighting. Cuttings were fertilized as needed with a slow-release fertilizer and were grown in the greenhouse until flowering began, at which time cuttings from male plants were identified and discarded. Female clones were used in crosses with either a resistant MO1 male or a susceptible ACR male as described above and shown in Figure 3.2. Crosses were again allowed to occur for approximately two months, or until the male plant had stopped producing viable pollen, in the interest of high seed production. After crossing,

females were harvested, dried at room temperature for at least two weeks, and seeds were then stratified as described above.

This cloning procedure was necessary to determine the genotype of the MO1 plants because waterhemp is a dioecious species, and thus two plants must be crossed for seed production to occur. If instead, waterhemp was a monoecious species, the plants could simply be allowed to self-pollinate, and the resulting progeny could be screened for glyphosate resistance. If progeny showed no segregation for resistance, then the inferred parental genotype would be homozygous for resistance (RR). In the case of waterhemp, however, the situation is more complex, for even if a MO1 plant is RR, it must be crossed with another plant to produce seed. If that plant is also RR, then no segregation for resistance would be observed in the progeny, and the seed would constitute a homozygous resistant line. However, if one of the parents were RR and the other Rr, and if glyphosate resistance were conferred by a single dominant gene, then the progeny from this cross would also show no segregation for glyphosate resistance, but the population would not be homozygous for resistance (the genotypes of the progeny would be RR and Rr).

Thus, in order to determine the genotype of a resistant plant, it must be crossed with a susceptible plant, but in order to obtain a homozygous resistant seed line, two resistant plants must be crossed (both with RR genotypes). The clones were utilized to potentially solve this problem in one generation—with the use of resistant female clones, a particular plant can be used in multiple crosses, with susceptible males to determine the genotype, and with resistant males (whose genotype is tested by including susceptible females in the cross) to potentially obtain homozygous resistant seed lines. Another potential solution to this problem would be to set up many crosses between resistant males (one male per cross) and multiple resistant females, which

need not be cloned. The progeny from such crosses could be screened with glyphosate, and if segregation was observed, both parents would be identified as Rr, and the seed from such crosses could be discarded. Lines tested that did not segregate for glyphosate resistance would have to contain either RR and Rr individuals or only RR individuals. To determine the genotypes of the seeds in such lines, multiple individuals from each line would need to be grown and then crossed with susceptible plants. The progeny from such crosses would then need to be screened, and if no segregation occurred after screening the progeny of multiple crosses involving parents from the same seed line (to ensure that at least one Rr plant, if any are present in the population, is crossed with a susceptible plant to produce detectable segregating progeny), then the seed line from which the resistant parents were obtained to create the non-segregating lines after crossing with a susceptible plant, could be identified as a homozygous resistant line. Thus, for waterhemp, to determine the genotypes of the resistant parents and produce homozygous resistant seed lines, the use of clones saves both time and a considerable amount of effort.

3.3.6 Initial evaluation of F_1 progeny

F_1 progeny were evaluated for resistance to glyphosate in order to gain insight into the mode of inheritance and level of dominance of glyphosate resistance. F_1 seed was sown from reciprocal crosses and plants were grown in the greenhouse as described above. MO1 and ACR were included as the resistant and susceptible controls, respectively. Also included were plants grown from seed collected from ACR x ACR crosses (pollen contamination tests) and MO1 x MO1 crosses. When plants reached 10–15 cm in height, they were treated with glyphosate at rates of 0, 210, 420, and 840 g ae ha^{-1}. The herbicide solution also contained 2.5% (v/v) AMS. Plants were evaluated for response to glyphosate at 14 days after treatment (DAT) and given a

visual rating corresponding to injury level on a 0 to 5 scale with 0 indicating no injury and a 5 indicating a complete kill. The same methods were later used to screen F_1 progeny created from clones, although plants were only treated with glyphosate at 0, 210 and 840 g ae ha^{-1}, which contained 2.5% (v/v) AMS. At 14 DAT, the plants were given ratings of resistant (R), intermediate (I), or susceptible (S), where R plants showed phenotypes similar to those of MO1, S plants showed phenotypes similar to ACR, and I plants showed a phenotype of a resistance level between those of ACR and MO1.

3.3.7 Initial creation of F_2 and backcrossed lines

F_1s surviving treatment with glyphosate from the most uniform line were selected and used to create F_2 and backcrossed (BC) lines. Crosses consisted of either an F_1 male or an ACR male, and all crosses included F_1 and ACR females, as well as female progeny from a MO1 x MO1 cross (Figure 3.4). The crosses were performed in either a growth chamber or in a pollination bag in the greenhouse as described above, and were allowed to continue for approximately two months in order to collect as much seed as possible. F_2 progeny were the result of crosses between F_1 plants, while backcrossed lines were obtained from crossing an F_1 plant to a susceptible ACR plant in either direction. As these backcrosses were made by crossing to a glyphosate-susceptible parent, the backcross lines will be abbreviated as BC_S (for *backcrossed to susceptible*). After crossing was complete, females were harvested and seed was collected and stratified as previously described.

3.3.8 Initial evaluation of F_2 and BC_S progeny

3.3.8.1 Herbicide screen

Seeds were sown from several F_2 and BC_S lines, as well as from an F_1 line. MO1 and ACR were sown to serve as resistant and susceptible controls, respectively. Plants were grown in the greenhouse as described above until they reached 10–15 cm in height, at which time they were screened with glyphosate in order to investigate segregation ratios of R:S, which could potentially indicate the number of genes responsible for conferring resistance. Plants were treated with glyphosate at rates of 0, 420, 840, 1680, and 3360 g ae ha^{-1}. The herbicide solution included 2.5% (v/v) AMS.

At 14 DAT, plants were classified as resistant (R), intermediate (I), or susceptible (S), depending on whether their response to glyphosate most closely resembled that of MO1, the F_1s, or that of ACR, respectively, and R:I:S ratios were determined. Results from separate F_2 lines and separate BC_S lines were tested for pooling and BC_S lines were pooled when possible based on results of a chi-square (χ^2) test for homogeneity across all lines of the corresponding type. Standard χ^2 tests were then performed using the observed segregation ratios to test the hypothesis that glyphosate resistance is conferred by a single dominant or partially dominant gene, in which case the expectations would be segregation ratios of 3:1 (R:S) or 1:2:1 (R:I:S) for F_2s and 1:1 (R:S) or 0:1:1 (R:I:S) for BC_S lines (Figure 3.5).

3.3.8.2 Growth on artificial media

Due to difficulty in differentiating between phenotypes of F_2s and BC_S plants treated with glyphosate, another attempt was made to analyze segregation ratios of these plants after growth on artificial media containing glyphosate. The artificial media mix consisted of 1% (w/v)

sucrose, a 1x concentration of Murashige and Skoog Basal Salt Mixture[8] (MS salts) (4.3 g L^{-1}), and 1% (w/v) agar[9]. Glyphosate[10] was added to the media at concentrations of 0, 33, 99, 296, 889, 2660, and 7990 µM. At each concentration of glyphosate, 100 ml of media was made and then poured into four separate petri plates (25 ml per plate) and allowed to cool.

Meanwhile, MO1, ACR, F_1 and BC_S seeds were sterilized by treatment in a 15% bleach/water solution containing 0.1% (v/v) sodium dodecyl sulfate (SDS) detergent for 15 min. Seeds were then rinsed three times with sterile deionized water and then placed in a top agar solution and poured onto the glyphosate-containing media in the petri plates. Top agar consisted of 1% (w/v) sucrose, a 1x concentration of MS salts, and 0.8% (w/v) agar. Plates were then covered and wrapped twice with parafilm[11] and placed in a growth chamber set at 28 C/22 C day/night and programmed for a 16-hour photoperiod. The growth response of the seedlings to glyphosate was recorded after 14 days in the form of qualitative observations of growth. All procedures involving the opening of the plates were performed inside a sterile fume hood to reduce potential for contamination of the media.

3.3.9 Investigation of relative copy number of *EPSPS*

Due to questions about the validity of data collected from the first run of the experiment (i.e., due to unexpected observations of segregation made in F_1s, F_2s and BC_S plants from the first run), the entire experiment was conducted again with some slight modifications. MO1 plants were screened for glyphosate-resistance as discussed previously. Resistant MO1 plants were selected, and the apical meristems removed in preparation for cloning. After sufficient growth from axillary buds, eight cuttings were taken from each of the MO1 plants and placed in the mist room. After root growth began, cuttings were potted and placed back in the original greenhouse

room. The original plants from which the cuttings were taken were also kept. These plants began flowering before the cuttings, allowing for the removal of male clones before flowering began—these plants were discarded. When the first of the remaining female clones began flowering, all clones were placed in a growth chamber, allowing flower branches to develop while awaiting male MO1 and ACR plants to begin flowering. Crosses were then conducted in the greenhouse in pollination bags consisting of either a resistant MO1 male or an ACR male, as well as MO1 female clones and an ACR female (Figure 3.2). Tissue samples of approximately 100 mg each were collected from all plants in the crosses, including the clone plants, and these samples were then stored at -80 C until needed.

After crosses were complete, females were harvested and dried, and seed was collected and stratified as previously described. The F_1 seed was then sown, and plants were grown until they reached 10–15 cm in height, at which time they were treated with glyphosate at rates of 0, 840, 1680, or 3360 g ae ha^{-1}. Herbicide treatments also contained 2.5% (v/v) AMS and 0.25% (v/v) nonionic surfactant (NIS)[12]. Plants were given visual ratings of injury level at 14 DAT on a scale of 0 to 10 based on comparison with the untreated control plants as well as the responses of MO1 and ACR, with 0 indicating a healthy plant, and a 10 indicating a complete kill.

In this run, F_1 male plants of various phenotypes from healthy to moderately injured were selected for crossing with F_1 female plants of various phenotypes to create F_2s with the constraint that crosses consist of full-sib matings. This was done based on results of a concurrent study conducted on glyphosate-resistant Palmer amaranth [*Amaranthus palmeri* S. Wats.], from which it was determined that resistance to glyphosate in that species is conferred by amplification of the gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), leading to increased expression of the enzyme (Gaines et al. 2010). It was thought that a similar mechanism may

confer resistance in waterhemp. Therefore, plants of various phenotypes were chosen in order to investigate the inheritance of suspected varying levels of gene amplification in this species. These crosses were performed in pollination bags in the greenhouse and were allowed to progress for approximately two months, after which time the females were harvested and dried and seed was collected and stratified. Also included were two ACR females, each placed in separate pollination bags in the greenhouse room in which F_1 crosses were being performed, as a test for pollen flow between pollination bags. Tissue samples were collected from the F_1 plants used as parents, as well as from other F_1 plants displaying a broad range of responses to glyphosate from resistant to susceptible. These samples were then stored at -80 C until needed. A second F_1 screen as performed in which F_1 plants from various lines were treated with glyphosate at 0, 1260, and 2520 g ae ha^{-1}. These plants were also given visual ratings at 14 DAT, and tissue samples were collected for gene amplification analysis.

Seeds from various F_2 lines were then sown and plants were grown in the greenhouse as previously described until they reached 10–15 cm in height, at which time they were treated with glyphosate at rates of 1680 and 4200 g ae ha^{-1}. The herbicide solution also contained 2.5% (v/v) AMS and 0.25% (v/v) NIS. Also included were plants from the F_1 parental lines, as well as from ACR and MO1 for comparison. Plants were given visual injury ratings on a 0–10 scale at 14 DAT. Once again, 100 mg tissue samples were collected from all plants at the time of treatment and were stored at -80 C until needed.

3.3.9.1 DNA extraction

Total genomic DNA (gDNA) was extracted from meristematic leaf tissue by using a modified hexadecyltrimethyl-ammonium bromide (CTAB) protocol from Doyle and Doyle

(1990), and the extracted DNA was resuspended in 50 µL of TE buffer. DNA was then quantified using a spectrophotometer[13] and was then diluted to 10–50 ng μL^{-1} using sterile deionized water. The DNA was then stored at -20 C until needed.

3.3.9.2 Optimization of quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed, generally following the methods given by Gaines et al. (2010) in order to investigate the possibility of *EPSPS* gene amplification as a glyphosate resistance mechanism in waterhemp. The choice of a suitable endogenous control gene for normalizing *EPSPS* copy number was decided following preliminary qPCR experiments (data not shown) that were designed to evaluate primer specificity, amplification efficiency, and copy-number stability. Ideally, the reference gene should be single-copy, well-conserved and stable in all biological samples, including resistant and susceptible biotypes. In addition to surveying the literature for single-copy genes in *Amaranthus* and related species, BLAST searches of plant single-copy orthologous genes were also conducted (Wu et al. 2006) against a custom in-house *Amaranthus* 454-derived expressed sequence tag (EST) library. Identities of putative single-copy genes in waterhemp were confirmed through high probability tBLASTx hits (> 90% identity) of EST unigenes and subsequent sequence alignments. Multiple sequence alignments for six candidate genes were produced to identify conserved exon regions of at least 100 bp in length to anchor primers.

Primers were designed using BatchPrimer3[14] and subsequently checked for self-complementarity, hairpins, and dimers using IDT OligoAnalyzer[15]. Of the six candidate reference genes, one termed 9240 was selected for further analysis based on qPCR experiments. This gene encodes the large subunit of carbamoylphosphate synthetase (CPSase) and shares

> 90% amino acid sequence identity to CPSases from other plant species, including *Arabidopsis* (At1g29900), *Nicotiana* (AJ319872), and *Medicago* (FJ388886). Another reference gene, acetolactate synthase (*ALS*), was also evaluated since it was used as the reference gene to demonstrate gene amplification as the mechanism responsible for glyphosate resistance in Palmer amaranth (Gaines et al. 2010).

One microliter aliquots of gDNA (10–50 ng μL^{-1}) were distributed individually on a 384-well plate, and each biological sample was done in triplicate. To each sample was added 9 μL of master mix containing 1x SYBR® Advantage® qPCR premix[16], 1x ROX LSR reference dye, 0.3 μM each primer[17], and molecular biology-grade water[18] for a final reaction volume of 10 μL. Newly designed primers for the reference gene were 9240qF (5'-ATT GAT GCT GCC GAG GAT AG-3') and 9240qR (5'-GAT GCC TCC CTT AGG TTG TTC-3'), and for the target gene were EPSqF2 (5'-GGT TGT GGT GGT CTG TTT CC-3') and EPSqR2 (5'-CAT CGC TGT TCC TGC ATT TC-3'). Amplicon sizes were 78 bp and 81 bp, respectively. For comparisons, amplifications of *ALS* and *EPSPS* were also performed using primers ALSF2, ALSR2, EPSF1, and EPSR8 described by Gaines et al. (2010). An ABI Prism® 7900 Detection System[19] was used for quantitative PCR with the following parameters: 50 C for 2 min, 95 C for 10 min, and 40 cycles of 95 C for 30 sec and 60 C for 1 min. Following the final qPCR cycle, a dissociation curve analysis (Figure 3.6) was implemented to monitor amplicon specificity by first holding the temperature at 95 C for 4 min, then decreasing to 55 C for 4 min, and then slowly ramping to 95 C. The dissociation peak for both control and target products was 78 C. The detection threshold (C_t) was set manually for all experiments.

Relative standard curves (Figure 3.7) for each gene were generated from a 5x dilution series (100ng, 20ng, 4ng, 0.8ng, and 0.16ng μL^{-1}) using a susceptible biotype designated Wayne

County Susceptible (WCS), previously described by Patzoldt et al. (2005), as the template. This dilution series was chosen based on experiments that demonstrated results within the dynamic range of the assay. Standard curves were generated for each plate to ensure consistency between runs. Amplification efficiencies were determined for each gene using the equation

$$E = [10^{-1/\text{slope}} - 1] * 100 \quad [1]$$

where the slope is obtained from the linear regression of C_t values plotted against template concentration. Standard curve plots had high correlation coefficients ($r^2 \geq 0.97$) and had slopes within the acceptable range of -3.6 and -3.1 (90–110% efficiency). An ideal slope of -3.32 corresponds to a theoretical maximum efficiency of 100% (i.e., the amount of PCR product doubles with each cycle) (Anonymous 2008).

Relative quantification of *EPSPS* (the target gene) compared with either 9240 or *ALS* (endogenous controls) was measured using the Comparative C_t ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001; Schmittgen and Livak 2008; Bubner and Baldwin 2004). Average ΔC_t values for each biological sample (run in triplicate) were calculated with the formula

$$\Delta C_t = [\text{avg } C_t \text{ (target)} - \text{avg } C_t \text{ (reference)}]. \quad [2]$$

A validation experiment was performed to determine whether PCR efficiencies of the two genes were approximately equal by plotting ΔC_t values against the log-transformed concentration of gDNA from each dilution. The absolute value of the slope was ≤ 0.1, which passes the test for equality of efficiencies. Following validation, ΔC_t values for every sample were normalized to WCS (the calibrator sample) and fold-changes calculated relative to this representative susceptible biotype.

Repeatability and precision of the qPCR assay was assessed via biological replicates (samples from the same individual plant or population), technical replicates (triplicates in the

same run and/or the same sample in different runs), multiple positive (R-biotypes) and negative (S-biotypes and non-template) controls, and multiple reference genes. As an additional measure of reliability in the assay, this qPCR protocol was used to examine *EPSPS* amplification among 21 confirmed glyphosate-resistant individuals of *A. palmeri* from the same population cited by Gaines et al. (2010). Results were in agreement with those reported in this study showing a 7- to 53-fold increase in *EPSPS* copy number in resistant *A. palmeri* plants when compared with susceptible biotypes.

To investigate variation in *EPSPS* copy number, qPCR experiments were conducted with known susceptible and resistant biotypes (confirmed by herbicide treatments) to estimate a range from which associated calculated fold changes of unknowns could be associated with being either resistant or susceptible. Assuming a single copy of 9240 (CPSase) per haploid genome and no *EPSPS* amplification, the expected gene ratio in glyphosate-susceptible plants should be close to 1:1. Likewise, a 1:1 ratio is also expected when comparing the two reference genes 9240 to *ALS*. Based on the preliminary qPCR experiments, it was determined that plants with *EPSPS* copy number fold changes of < 2 could be considered susceptible, while plants with fold changes of > 3 were resistant to glyphosate (data not shown).

3.4 Results and Discussion

3.4.1 Initial F_1 screen

In the initial F_1 screen, plants from three different F_1 lines, as well as from ACR, test cross progeny, MO1, and MO1 x MO1 progeny were treated with glyphosate at rates of 0, 210, 420, and 840 g ae ha^{-1}. The results of this screen were nearly as expected. All three of the F_1 lines screened showed some level of glyphosate resistance (Figure 3.8). One of the lines tested

appeared to be nearly uniform for glyphosate resistance when applied at a rate of 420 g ae ha^{-1} (0.5 times the field use rate), with plants in this line displaying an intermediate (I) phenotype compared with those of MO1 and ACR, although some segregation was apparent at the 840 g ae ha^{-1} dose (data not shown). The other two F_1 lines showed more pronounced segregation for glyphosate resistance at all doses, and this segregation is clear in Figure 3.8. One of these two lines originated from a cross between a MO1 female with an ACR male (as did the previously mentioned nearly uniform F_1 line), while the other originated from a cross between an ACR female and a MO1 male. The fact that all three of the F_1 lines, representing reciprocal crosses between resistant and susceptible plants, contained individuals surviving treatment with glyphosate indicates that glyphosate-resistance is a nuclear inherited trait.

Close inspection of the susceptible controls reveals that ACR was well controlled by glyphosate. The test cross progeny (ACR x ACR) for the most part were even more susceptible to glyphosate than was ACR, which may be indicative of some effects of inbreeding depression. However, two of the test cross progeny survived treatment with glyphosate, as can be seen in the photograph. These plants appeared only slightly more injured than the F_1 plants from the near-uniform line, which may indicate a low level of pollen contamination in some of the crosses. If these surviving plants were due to pollen contamination, it was thought that this could potentially also explain the observed segregation in the three F_1 lines. Interestingly, the progeny from the MO1 x MO1 crosses (the three rightmost rows of plants in Figure 3.8) showed even less glyphosate injury than did MO1 at the 420 g ae ha^{-1} rate, displaying little or no yellowing of the meristems, unlike many of the plants from the parental MO1 population. However, these plants also appeared to display some evidence of inbreeding depression, as even the untreated control

plants from such crosses were noticeably shorter than the majority of the MO1 plants (data not shown).

Plants at all doses in this initial screen were given visible injury ratings on a scale of 0 to 5, with 0 indicating a healthy plant, and a 5 indicating a complete kill, and the mean visual ratings for several of the populations screened were plotted against glyphosate dose in Figure 3.9. The ACR plants and the test cross progeny displayed high levels of glyphosate injury even at the lowest dose, as can be seen clearly in the figure. This figure again shows that MO1 x MO1 progeny demonstrated a high level of resistance to glyphosate, with a mean visual injury rating of nearly 0 at the 210 g ae ha^{-1} dose, compared with 1.5 for MO1 at the same dose. The F_1 lines showed injury levels similar to those of MO1 plants at the 210 g ae ha^{-1} dose, but at higher doses displayed a mean visual rating between that of MO1 and ACR. The fact that all three of the F_1 lines show an intermediate level of injury at various doses of glyphosate again indicates that glyphosate resistance is a nuclear inherited trait. Thus, based on the results of this screen, the modified hypothesis was that glyphosate resistance is a nuclear-inherited, partially dominant, single gene trait.

3.4.2 Initial screening of F_2 and BC_S lines

Based on the results of the initial F_1 screen, glyphosate-resistant individuals from the nearly uniform line were selected for use in creating F_2 and BC_S lines, which were produced by crossing the most resistant F_1 plants either with other resistant F_1 plants or with ACR, respectively. Seed was collected from the females in these crosses, and F_2 and BC_S plants were grown as previously described. When these plants reached 10–15 cm in height they were screened for glyphosate resistance, as were MO1, ACR, and F_1 plants. The two lowest rates

failed to adequately control susceptible plants in this run of the experiment, so plants treated with glyphosate at the two highest rates (1680 and 3360 g ae ha^{-1}, corresponding to 2 and 4 times the field use rate) were analyzed for segregation in order to determine the number of genes responsible for conferring resistance to glyphosate. 5 BC_S lines and 3 F_2 lines were screened in this experiment.

After recording the results of the herbicide screening, the BC_S lines were subjected to a χ^2 test for homogeneity, which they passed with a p-value of 0.997 at the low dose, and with a p-value of 0.191 at the high dose and thus were pooled for both doses of glyphosate. Expected segregation ratios in the BC_S lines, based on the partially dominant single gene hypothesis, were 0:1:1 (R:I:S). Observed segregation ratios were compared with expectations, and a χ^2 test was performed to determine whether the observed segregation ratios fit a single gene model (Table 3.1). However, with the expectation that half of the BC_S plants would be intermediate (I) and half would be S, the calculated χ^2 values were 16.1 and 45.5 corresponding to p-values of < 0.0001 for both doses. This indicated that a single gene model did not describe the data. However, closer examination of the table reveals the fact that not all F_1 plants displayed the intermediate phenotype—in fact some F_1 plants were killed at both doses.

It was decided that, because of the death of supposed intermediate F_1 plants, the segregation expectations for the BC_S plants should be modified. At the 1680 g ae ha^{-1} rate, 5 out of the 18 F_1 plants expected to display an intermediate phenotype were killed. Thus the expectations for the BC_S plants now were that, of the half of the BC_S plants originally expected to display an intermediate phenotype, 5/18 (or 28%) of them would actually display a susceptible phenotype. Therefore, 28% of the originally expected intermediate plants were added into the expected susceptible category, giving new expectations of 41.5 intermediate and 73.5 susceptible

BC_S plants out of a total of 115 screened at the 1680 g ae ha^{-1} dose (Table 3.2). The expectations for BC_S segregation at the 3360 g ae ha^{-1} dose were similarly modified based on the responses of F_1 plants at this dose, in this case with 8/18 (or 44%) of originally expected intermediate plants showing a susceptible phenotype. Thus, the expected ratio of I:S for the 114 BC_S plants screened at the high dose of glyphosate became 31.7 : 82.3 rather than the originally expected 57:57 (or 1:1).

The modification of expectations did improve the fit of the BC_S plants' segregation to that expected in the case of a single partially dominant gene at the 1680 g ae ha^{-1} dose of glyphosate, giving a χ^2 value of 1.2, with a corresponding p-value of 0.273. However, despite the correction made at the higher dose, the observed segregation ratios still failed to fit a single gene model at the 0.05 confidence level, with a χ^2 value of 5.0 and a corresponding p-value of 0.025.

Based on the observations made in analysis of the BC_S plants, it appeared that there was some overlap between the susceptible and intermediate phenotypes. This problem was avoided altogether in the F_2 analysis by grouping plants in the intermediate and susceptible categories together into one large phenotypic class (called S). With this new grouping, in the case of a single partially dominant gene, the expected segregation ratios for the F_2 lines became 1:3 (R:S). A χ^2 test for homogeneity revealed that the three F_2 lines screened in this experiment could be pooled at the lower dose of glyphosate, with a p-value of 0.162, although at the higher dose the F_2 lines failed the homogeneity test, with a p-value of 0.010. Therefore, the F_2 lines were analyzed separately at both doses, but were also pooled and analyzed at the 1680 g ae ha^{-1} dose (the low dose) for comparison.

Table 3.3 shows the results of the F_2 screen. At the low dose, all three of the F_2 lines passed the χ^2 test for a single partially dominant gene. One of the lines (MBX18) fit the

expectations perfectly, with 15 R plants and 45 S plants, which gave a χ^2 value of 0 and a corresponding p-value of 1. The other two lines passed the test with p-values of 0.233 and 0.136. When these three F_2 lines were pooled at this dose, the group of all F_2s also passed a χ^2 test for a single partially dominant gene, with a p-value of 0.863 (data not shown).

At the higher dose of glyphosate, however, the results were different, with only one out of the three F_2 lines fitting a single gene model with a p-value of 0.371, while the other two lines failed to fit the model at the 0.05 confidence level, with p-values of 0.036 and 0.043. Inspection of Table 3.3 reveals that the reason for the failure of the F_2 lines to fit the single gene model at the high rate of glyphosate is that fewer plants displayed the R phenotype than were expected. A first thought as to the reason for this is that perhaps the glyphosate rates used were too high. However, the resistant and susceptible controls behaved as expected at both doses, and thus the problem should not have been with the glyphosate doses applied. Thus, the results of this initial screen were inconclusive. It was thought that perhaps another explanation for the problem may lie in the inherent difficulty in distinguishing among phenotypes after glyphosate treatment (see Appendix A for a related discussion), and so an alternative approach to analyzing segregation was attempted.

3.4.3 Screening on artificial media

Phenotypes of ACR, MO1, F_1s, and BC_S plants were analyzed after germination in glyphosate-containing artificial media, with qualitative characteristics recorded at two weeks after seeds were placed on the plates, in the hope that clear segregation among phenotypes would be observed. On the plates containing no glyphosate, the growth of seedlings from all populations was similar. Seedlings had fairly long, branched roots and green shoots, usually with

two true leaves. On plates containing 33 µM glyphosate, some root growth inhibition was visible on 2/3 of the ACR seedlings, with the remaining 1/3 showing root growth similar to that on plates with no glyphosate in the media. However, the shoots of these ACR seedlings still appeared healthy—they were green with two true leaves. MO1 at this dose showed little or no root growth inhibition, and grew healthy shoots. At this dose, 2/9 and 2/4 of the F_1s and BC_S seedlings, respectively, appeared to show signs of root growth inhibition, although it was unclear whether this was due to the presence of the herbicide or to late germination. Shoots of these seedlings appeared healthy, with two true leaves.

At the 99 µM dose, ACR seedlings showed clear injury, with severely stunted root growth and yellow cotyledons that had barely unfolded. The shoots of these plants were also noticeably shorter than those of all seedlings at the lower doses, being only approximately 5 mm tall compared with approximately 10–20 mm for seedlings at lower doses. MO1 seedlings showed signs of slight inhibition of root growth at this dose, although the shoots were green with two true leaves. The F_1 seedlings surprisingly showed less root growth inhibition than MO1, perhaps due to the fact that the F_1 seeds germinated earlier than MO1. There appeared to be some segregation in shoot growth of the F_1s, with 5/13 showing only cotyledons, which varied from yellow to green, and the remaining 8/13 displayed two true leaves, with those on 5 of the seedlings being green and on the other 3 yellow. The BC_S plants appeared to segregate for root and shoot growth at this dose, with 4/11 showing normal root growth, and the remaining 7/11 showing roots like ACR. Four of these plants displayed only cotyledons, while the remaining 7 had 1–2 true leaves, which were green in 6 plants and yellow in 1.

At the 296 µM dose, ACR seedlings barely grew; root growth was severely inhibited, and the shoots displayed very light yellow cotyledons which barely grew above the top of the agar.

MO1 at this dose displayed limited root growth, but shoots grew to the top of the plate, and all plants had begun to show signs of producing true leaves, although some yellowing was visible in these developing leaves. The F_1 plants again showed some segregation, with the healthiest seedlings showing root growth like that of ACR at the 33 µM dose, and the rest showing severe inhibition of root growth. The shoots of these plants did grow, but only produced 1 true leaf. 4/17 of the plants were green, while the remaining 13/17 showed considerable yellowing in the shoots and were typically shorter than the healthier seedlings. The BC_S seedlings also showed segregation at this dose, with phenotypes ranging from MO1-like to ACR-like.

At the 889 µM dose, ACR seeds germinated, but growth completely stopped soon afterward, with plants showing yellow cotyledons which had barely unfolded. The total plant length including root and shoot was approximately 5 mm for these seedlings. MO1 showed severely stunted roots, and all but one seedling appeared very yellow. Shoots of MO1 grew to about half the height of the plates (5–10 mm). The F_1s showed limited segregation at this dose, with 2 showing phenotypes similar to that of ACR, while the remaining 14 plants looked similar to MO1. As for the BC_S plants, all 7 of them looked similar to MO1.

At the 2660 µM dose, no growth occurred in ACR seedlings after germination. Cotyledons were a yellowish white color and they barely unfolded. MO1 seedlings showed severely inhibited root growth, although shoot growth was similar to that at the previous dose, except that at the current dose the shoots ranged in color from yellowish white (like ACR) to a greenish yellow. F_1 seedlings segregated, with 2 showing phenotypes like that of ACR, and the remaining 6 looking like MO1. The BC_S seedlings also segregated, with 4 appearing to show the ACR phenotype, and 3 looking like MO1.

At the highest dose tested (7990 µM), ACR was extremely injured, with all plant parts being completely white, and cotyledons in some cases failing to emerge from the seed coat. These seedlings failed to grow after germination. The MO1 seedlings were not much different than those of ACR at this dose, with very little growth occurring after germination, although all cotyledons did emerge from the seed coats, and a slight hint of yellow color was visible in the otherwise white MO1 seedlings. Because distinguishing R from S at this dose was nearly impossible, phenotypes of the F_1 and BC_S seedlings were not recorded.

The results of this study showed that segregation was indeed visible in the F_1 and BC_S lines grown on artificial media. However, like the results of the greenhouse herbicide screening, the phenotypes of plants grown on the artificial media were frequently difficult to separate into distinct categories, and it was determined that this experiment was not worth repeating. However, if, in the future, such a study must be performed, the results of this study show that the optimal dose for distinguishing R from S plants is likely somewhere between 99 and 296 µM of glyphosate.

3.4.4 Screening of F_1s involving clones

F_1 families created from the cloning experiment were screened for glyphosate resistance with the hope of finding at least one R_C x S family and one S x R family demonstrating uniform resistance to glyphosate, which would indicate that the resistant parents had been homozygous for glyphosate resistance in the case of the resistance being a single gene trait. Assuming successful identification of non-segregating families from crosses performed in both directions, seeds collected from the R_C x R cross involving two of the identified homozygous resistant parents would provide a uniform homozygous resistant line for use in future work. However, the

results of the F_1 screen in this experiment were similar to those seen in the initial F_1 screen, in that each of the 12 lines screened showed some level of segregation for glyphosate resistance (Table 3.4). In an extreme case, only 1 out of 10 F_1 plants survived glyphosate treatment at 210 g ae ha^{-1}. The test cross progeny were highly susceptible to glyphosate, again perhaps showing signs of inbreeding depression. Figure 3.10 shows photographs of segregation in several of the F_1 lines tested in this experiment, as well as the response of the test cross progeny to glyphosate at this dose. Based on the segregation again observed in F_1 lines, the conclusion was now that glyphosate resistance may not be a single gene trait. However, in order to be sure, it was decided to repeat this experiment, beginning with the creation of F_1 lines.

3.4.5 A second round of inheritance studies

During the preparation for the creation of the F_1s for the second round of this experiment, it was discovered that the mechanism of glyphosate resistance in the related species, Palmer amaranth, was due to elevated copy number of *EPSPS* (Gaines et al. 2010). It was hypothesized that, based on the results of the first run of the experiment, the same mechanism may be responsible for conferring glyphosate resistance in waterhemp. The second round of the experiment was thus conducted with this hypothesis in mind, and tissue samples were collected from all parents used in crosses.

3.4.5.1 Observations on F_1s and creation of F_2s

F_1 screens in the second round of this experiment gave results similar to those observed in the previous run, with all lines tested showing segregation for glyphosate resistance. Tissue samples were collected from numerous F_1 plants displaying a range of phenotypes in response to

treatment with glyphosate. Each plant was also given a visible injury rating between 0 and 10, with a 0 indicating a healthy plant, and a 10 indicating a complete kill. F_1s were selected for crossing to create F_2s based on their response to glyphosate, with the constraint that crosses utilized in creating F_2 plants consist of full-sib matings. Male plants showing a range of phenotypes from resistant to susceptible were selected for creation of F_2 lines, and where possible, F_1 females of several phenotypes were included with each of the male plants.

Tests for pollen contamination in this run consisted of placing individual ACR females in pollination bags that did not contain a male plant. These plants were then tested for seed production, which would indicate that foreign pollen had entered the pollination bags. Two such tests were performed, with approximately 50 seeds recovered from one plant, and approximately 200 seeds recovered from the other plant. With only 50 seeds collected from one of these plants, it is possible that the seed was produced from pollination that may have occurred before the female plant was isolated from males. However, in the case of the plant that produced approximately 200 seeds, at least some of this seed production was likely due to foreign pollen entering the pollination bag. Whether this happened during watering, or by airborne pollen grains travelling through the small holes in one pollination bag and then through the small holes in one of the pollination bags containing only a female plant is unknown. With seed production on the order of thousands for females in the desired crosses, it is estimated that in the worst case, perhaps 10% of seeds recovered from a particular desired cross could have been due to the presence of foreign pollen. However, in most cases seed production is so high that the percentage of seed produced due to pollen contamination would have been closer to 5% or less.

3.4.5.2 F_1 EPSPS relative copy number investigation

F_1 plants were tested for the relationship between response to glyphosate and *EPSPS* copy number relative to a susceptible WCS plant using qPCR as described above. Based on results of preliminary runs of qPCR, susceptible control plants (ACR) were found to consistently show relative copy numbers close to 1, as expected, when compared with WCS. Plants from the MO1 population consistently showed an elevated relative copy number, generally between 3 and 5 times that of the susceptible control (data not shown). Figure 3.11 shows a comparison of visual rating and relative copy number for susceptible and resistant controls, as well as for 74 F_1 plants from 7 different families treated with glyphosate at 1260 g ae ha^{-1}. This figure shows a weak relationship between visual rating and relative *EPSPS* copy number, with an R^2 value of 0.04 and a corresponding p-value of 0.07.

Immediately obvious from this figure is the fact that elevated *EPSPS* copy number is not sufficient to confer resistance to glyphosate in waterhemp. Although it appears that, for MO1, the higher the copy number the less injured the plant, this is not true for the F_1s, as plants with copy numbers as high as approximately 5 received injury ratings of 9, indicating that these plants were nearly killed by glyphosate. While it is true that plants with copy number similar to that of the susceptible control population also showed injury levels comparable with those of ACR, there are enough cases of F_1 plants having elevated copy number and still being killed by glyphosate to provide strong evidence that another factor must be necessary for conferral of resistance to glyphosate.

Inheritance of copy number in the F_1s was rather interesting, and this is shown in Figure 3.12. Although the susceptible plants generally show relative *EPSPS* copy numbers close to 1, as previously mentioned, the MO1 population generally shows a range of copy numbers (rather

than one value) in plants of approximately 3–5 times that of WCS (and ACR). Thus, relative copy numbers of parents used to create F_1 lines usually were not the same from one family to another, and thus the inheritance of copy number in the F_1s cannot be shown for all families simultaneously without some sort of normalization. This was performed by looking at the difference between the copy number of an F_1 plant of a particular family and the copy number of its susceptible parent. This difference in copy number was then compared with the difference in copy number between both the resistant and susceptible parents used to create that particular F_1 plant, in order to calculate inheritance of copy number as a percentage of the difference between those of the resistant and susceptible parents, as in

$$P_{ij} = \frac{C_{ij} - S_j}{R_j - S_j} \times 100\% \qquad [3]$$

where P_{ij} is the percentage of difference in copy number between the R and S parents that was inherited by the i^{th} F_1 plant examined from the j^{th} F_1 family, C_{ij} is the calculated relative copy number of the i^{th} F_1 plant examined from the j^{th} family, S_j is the calculated copy number of the susceptible parent of the j^{th} family, and R_j is the calculated relative copy number of the resistant parent of the j^{th} family. In this way, inheritance of copy number in all F_1 plants may be displayed simultaneously. Families were also separated by whether the resistant plant acted maternally or paternally, but as no difference was observed between these two types of analyses, all plants tested from both directions of crosses were included in Figure 3.12.

Investigation of the figure, which represents data collected from 28 plants originating from 5 different F_1 families, shows that F_1 plants inherited any number of copies of *EPSPS* from the parents from a number similar to that contained in the susceptible parent to even more copies than were contained in the resistant parent. The reason for this type of inheritance is not clear, but it seems to imply that copy number is inherited as a quantitative trait, perhaps indicating that

extra copies of *EPSPS* are distributed throughout the genome. If these copies were located near each other on the same chromosome, the inheritance would be expected to look more like that of a single gene trait, with F_1 plants inheriting half of the difference in copy number between the R and S parents. That copies of *EPSPS* may be found on multiple chromosomes has been previously shown for Palmer amaranth (Gaines et al. 2010). It would be interesting to perform Southern blot analysis on *EPSPS* in waterhemp, which could potentially be used to test this hypothesis of the copies of *EPSPS* also being located on multiple chromosomes in this species.

3.4.5.3 Observations on F_2s

Based on the results of experiments conducted to this point in the study, segregation ratios were not analyzed in the newly created F_2 populations, although these populations were indeed observed to segregate for glyphosate resistance. Before screening the F_2s created in this round of the experiment, tissue samples were collected from all plants in order to again investigate the relationship between glyphosate response and *EPSPS* copy number. Plants were then screened for glyphosate resistance at rates of 1680 and 4200 g ae ha^{-1}. At 14 DAT plants were given visual injury ratings and lines found to display the widest range of phenotypes were selected for use in qPCR.

3.4.5.4 F_2 EPSPS relative copy number investigation

F_2 plants receiving both doses of glyphosate were used for qPCR analysis, and their parents were also included in order to investigate the inheritance of *EPSPS* relative copy number. Figure 3.13 shows the relationship, at the 1680 g ae ha^{-1} dose of glyphosate, between injury level and relative copy number, which is weak at best, with an R^2 value of 0.02. This figure shows that

plants with relative copy numbers of nearly five times that of the susceptible control plants were killed, while other plants—most notably a plant for which the calculated relative copy number was less than that of the susceptible control plants—survived glyphosate with a rating of only 2, indicating that the plant showed very mild symptoms of glyphosate injury at this dose. Thus, in the case of the F_2 populations, it begins to appear that copy number and resistance level are possibly segregating independently.

Figure 3.14 shows the same relationship for plants that were sprayed with glyphosate at a rate of 4200 g ae ha^{-1}. Again the correlation between visual injury and copy number is weak, although, presumably due to the higher rate of glyphosate applied, all plants with relative copy number of less than 3 showed injury ratings of at least 4, unlike some plants sprayed at the 1680 g ae ha^{-1} rate that displayed lower injury levels with the same relative copy number of *EPSPS*. However, the relationship between relative copy number and resistance level remains unclear. From the F_2 data, again it seems that, although gene amplification may be important in conferring resistance to glyphosate, it is most definitely not sufficient to confer resistance, as plants with elevated copy number are often killed by glyphosate.

Figure 3.15 shows the inheritance of relative copy number of *EPSPS* in the F_2s for 88 plants originating from 5 different families. Again due to varying levels of relative copy number between the parents of each family, inherited copy numbers of F_2 plants were normalized to the difference in copy number between the parents. The result is quite interesting, showing that approximately 70% of F_2 plants show relative copy numbers that fall outside of the range of those of the parents. This appears to be similar to the effects observed in cases of transgressive segregation, which are often observed in the inheritance of quantitative traits (Sleper and

Poehlman 2006), although the authors report that plants falling outside of the range of the parents are seen in the F_3 generation.

It is difficult to draw many conclusions from the results of this study, although several may be drawn safely. Glyphosate resistance has been found to be a nuclear inherited trait, as resistant or intermediate F_1 plants can be obtained from crosses between MO1 and ACR performed in either direction. The trait is either dominant or partially dominant, although the exact level of dominance is difficult to quantify due to segregation in the F_1s and difficulty distinguishing between resistant and intermediate phenotypes. Based on observed segregation ratios in F_2 and BC_S lines, the trait appears to be nearly inherited as a single gene at lower rates of glyphosate, but not at higher rates. Investigations into the role of *EPSPS* gene amplification in conferring resistance to glyphosate have indicated that although it may be somehow involved, gene amplification is not the only necessary factor, and that the other factor, if it exists, may actually be more important in conferring resistance. This finding is not surprising based on work performed on another glyphosate-resistant population from Illinois discussed in Chapter 5, in which no gene amplification is observed in any plants from the resistant population. However, the investigation into the inheritance and mechanism of glyphosate resistance in the Missouri population is far from over. Future work could include investigations into the level of glyphosate absorption and translocation in MO1 compared with susceptible populations, as well as investigations into possible metabolism of glyphosate within plants.

3.5 Sources of Materials

[1] LC1 professional growing mix, Sun Gro Horticulture Canada Ltd., 52130 RR 65, P.O. Box 189, Seba Beach, AB 70E 2BO Canada. Distributed by Sun Gro Horticulture Distribution Inc. 15831 N.E. 8th St., Suite 100, Bellevue, WA USA 98008.

[2] Scotts Osmocote Classic 13-13-13 slow-release fertilizer. The Scotts Company LLC, 14111 Scottslawn Rd., Marysville, OH 43041.

[3] TeeJet 80015EVS spray nozzle. TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187.

[4] Roundup WeatherMAX® Herbicide. Monsanto Company, St. Louis, MO 63167.

[5] N-Pak® AMS Liquid, Winfield Solutions, LLC, P.O. Box 64589, St. Paul, MN 55164-0589.

[6] 78" x 72" 1.75 mil Pollination Bags. Vilutis & Co., Inc. 1135 Center Rd., Frankfort, IL 60423.

[7] Rootone® rooting hormone with fungicide. GardenTech™. P.O. Box 24830, Lexington, KY 40524-4830.

[8] Murashige and Skoog Basal Salt Mixture. Sigma Chemical Co. P.O. Box 14508 St. Louis, MO 63178.

[9] Bacto™ Agar. Becton, Dickinson and Company. Sparks, MD 21152.

[10] MON 76255 40.2% ae Technical Grade Glyphosate. Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167.

[11] Parafilm. Pechiney Plastic Packaging. Menasha, WI 54952.

[12] Activator 90 Nonionic Surfactant. Loveland Products, Inc. PO Box 1286, Greeley, CO 80632.

[13] Nanodrop 1000 Spectrophotometer v3.7.1. Thermo Fisher Scientific Inc., 81 Wyman St., Waltham, MA 02454.

[14] BatchPrimer3. http://wheat.pw.usda.gov/demos/BatchPrimer3/

[15] IDT OligoAnalyzer. Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241. http://www.idtdna.com/analyzer/applications/oligoanalyzer/

[16] 1x SYBR® Advantage® qPCR premix. Clontech Laboratories, Inc. 1290 Terra Bella Ave., Mountain View, CA 94043.

[17] IDT Custom Oligos. Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241.

[18] AccuGENE® Molecular Biology Grade Water. Lonza. Rockland, ME.

[19] ABI Prism® 7900 Detection System. Applied Biosystems. 850 Lincoln Centre Drive, Foster City, CA 94404 USA.

3.6 Acknowledgements

Thank you to Dr. Chance Riggins who spent much time in optimization of the qPCR and who helped to write a significant portion of the qPCR optimization section of this chapter. Thank you to Dr. Kevin Bradley for providing the seed from the MO1 population, without which, this project would not have been possible. Thanks also to Dr. Sukhvinder Singh who helped at least twice with taking cuttings from MO1 females for the cloning aspect of this project. Thank you to Dr. Ryan Lee who provided me with much-needed assistance during the experiment involving the use of artificial media. And thank you to Dr. Patrick Tranel who helped to guide me through the necessary steps in this study, particularly in the early phases when my knowledge on genetics was somewhat lacking.

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3.8 Tables and Figures

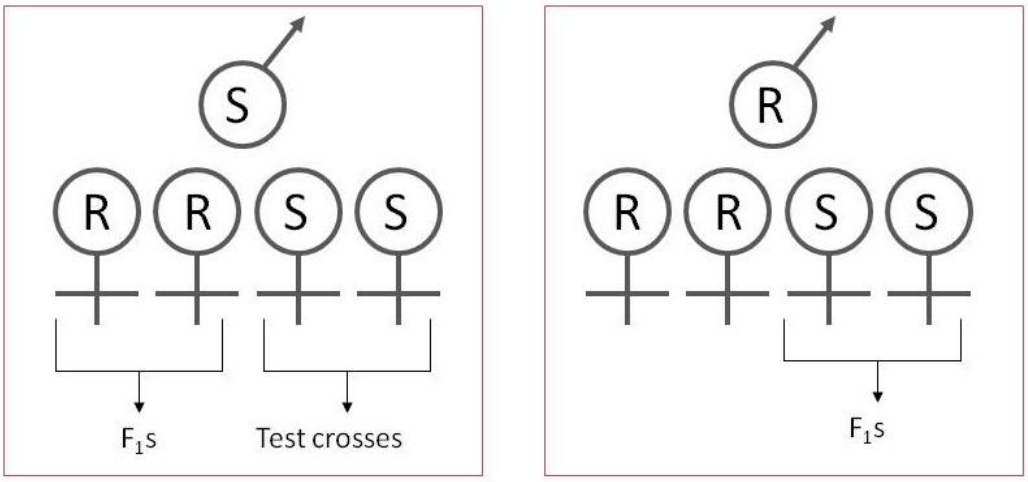


Figure 3.1 Diagrams of crosses utilized to produce F_1 seeds. S indicates a susceptible ACR plant, while R indicates a resistant MO1 plant. Susceptible females were included in crosses with susceptible males as a test for pollen contamination, and these are labeled as test crosses. Progeny from such crosses was expected to be susceptible to glyphosate in the absence of contamination with R pollen.

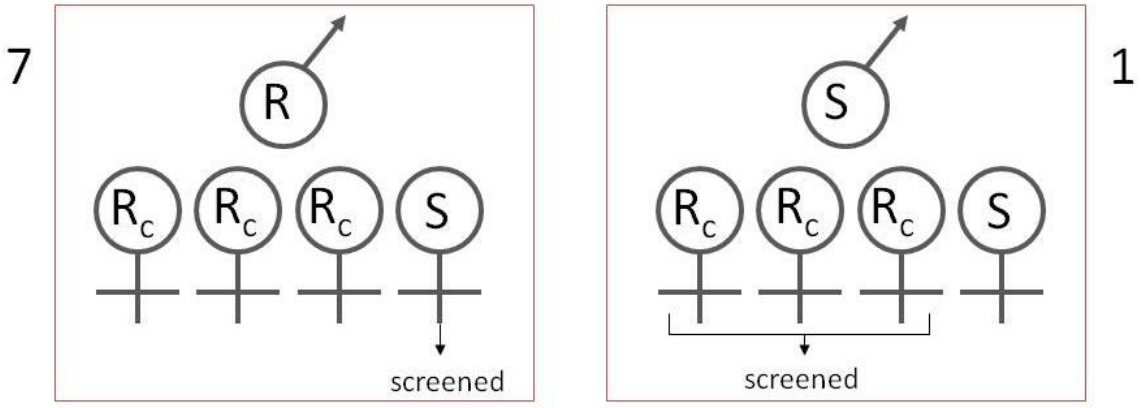


Figure 3.2 Diagram of crosses utilized to produce F_1s and homozygous glyphosate-resistant lines by using clones. R indicates a resistant MO1 plant, while S indicates a susceptible ACR plant. Clones are indicated by R_C (for Resistant Clone). Eight clones were made from each of multiple MO1 females, and one clone from each of the females was included in each of eight crosses. An S female was included in the cross with the S male as a test for pollen contamination, in the absence of which all progeny from this cross should be susceptible to glyphosate. R_C x S (♀ x ♂) progeny were screened for uniformity in response to glyphosate, which would indicate a homozygous resistant female plant. S x R (♀ x ♂) progeny were screened for uniformity in glyphosate resistance to attempt to identify a homozygous resistant male. Assuming both male and female homozygous resistant MO1 plants could be identified through these screens, seed collected from an R_C x R cross involving both of these parents should be uniformly resistant to glyphosate.

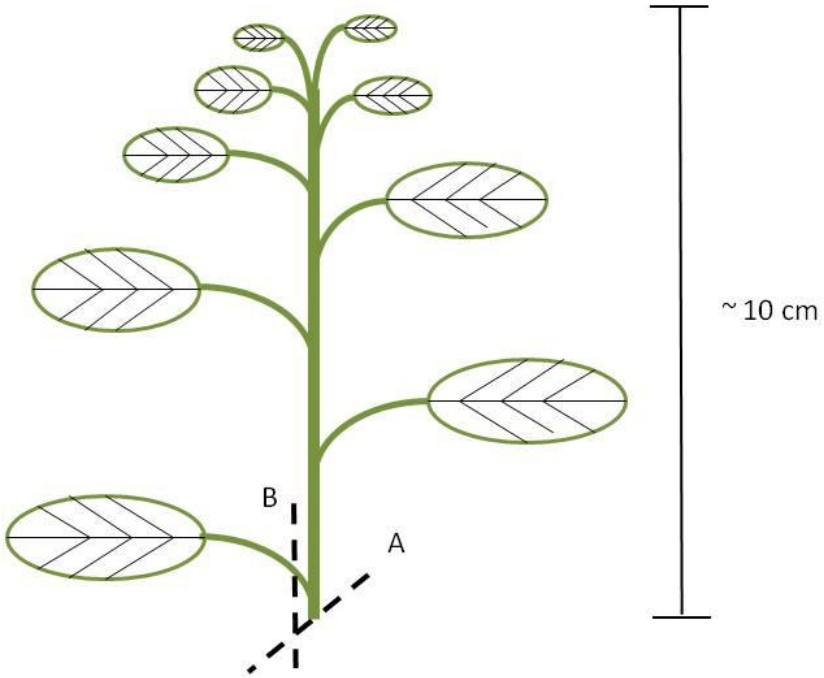


Figure 3.3 Figure depicting the top 10 cm of a branch of a glyphosate-resistant MO1 female used for cloning. Branches for were cut at an angle directly below a leaf (A) at approximately 10 cm in length to create cuttings. The bottom leaf from the newly cut branch was then excised (B). The area from which the leaf was removed was coated in root growth hormone and placed in damp soil in a mist room for approximately two weeks, until adequate root growth had occurred, at which time the cuttings were potted and grown for use in crossing.

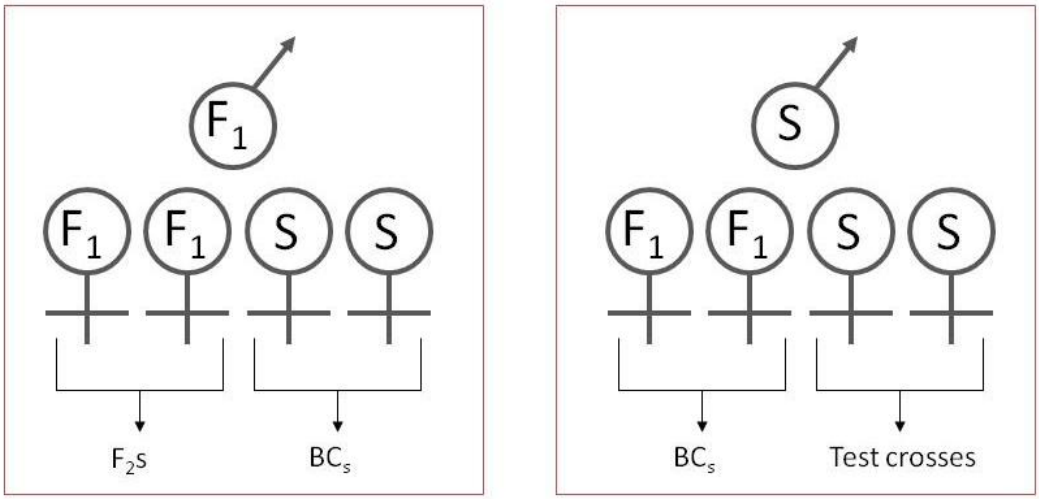


Figure 3.4 Diagrams of crosses utilized to create F_2s and BC_S lines, where S indicates a susceptible ACR plant. F_2s were produced from crosses involving an F_1 male with an F_1 female. BC_S lines were produced by crossing a susceptible ACR plant with a resistant F_1 plant in either direction. S plants were included in crosses involving an S male as a test for pollen contamination, in the absence of which all progeny from such a cross should be susceptible to glyphosate.

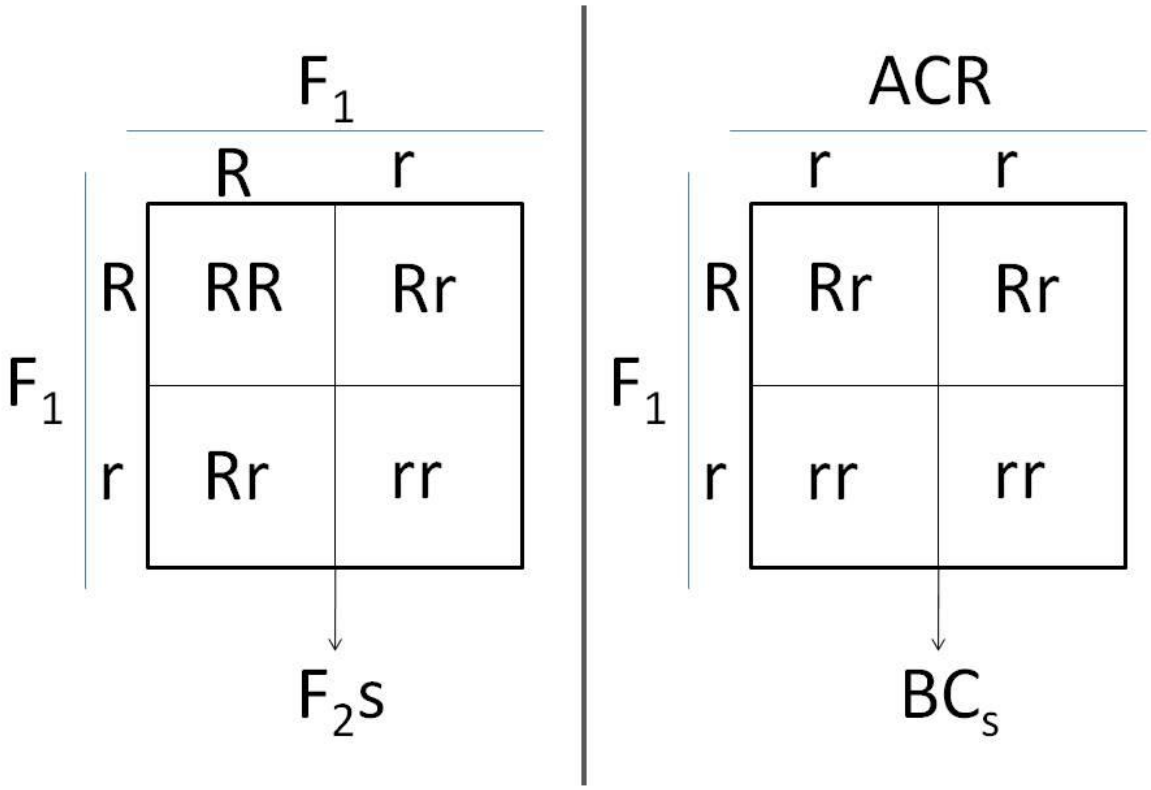


Figure 3.5 Diagrams of genotypes expected from crossing an F_1 with an F_1 to create F_2s, or from crossing an F_1 with a susceptible ACR to create BC_S lines. If glyphosate resistance were a single gene dominant trait, genotypes of RR and Rr should correspond to resistant phenotypes, and rr should correspond to the susceptible phenotype. Thus the expected segregation ratios would be 3:1 (R:S) in the F_2s and 1:1 in the BC_S lines. If glyphosate resistance were a single gene partially dominant trait, the RR genotype would correspond to the resistant phenotype, Rr would correspond to an intermediate level of resistance (I), and rr should be the susceptible genotype. In this case the expected segregation ratios would be 1:2:1 (R:I:S) in the F_2s and 0:1:1 in the BC_S lines.

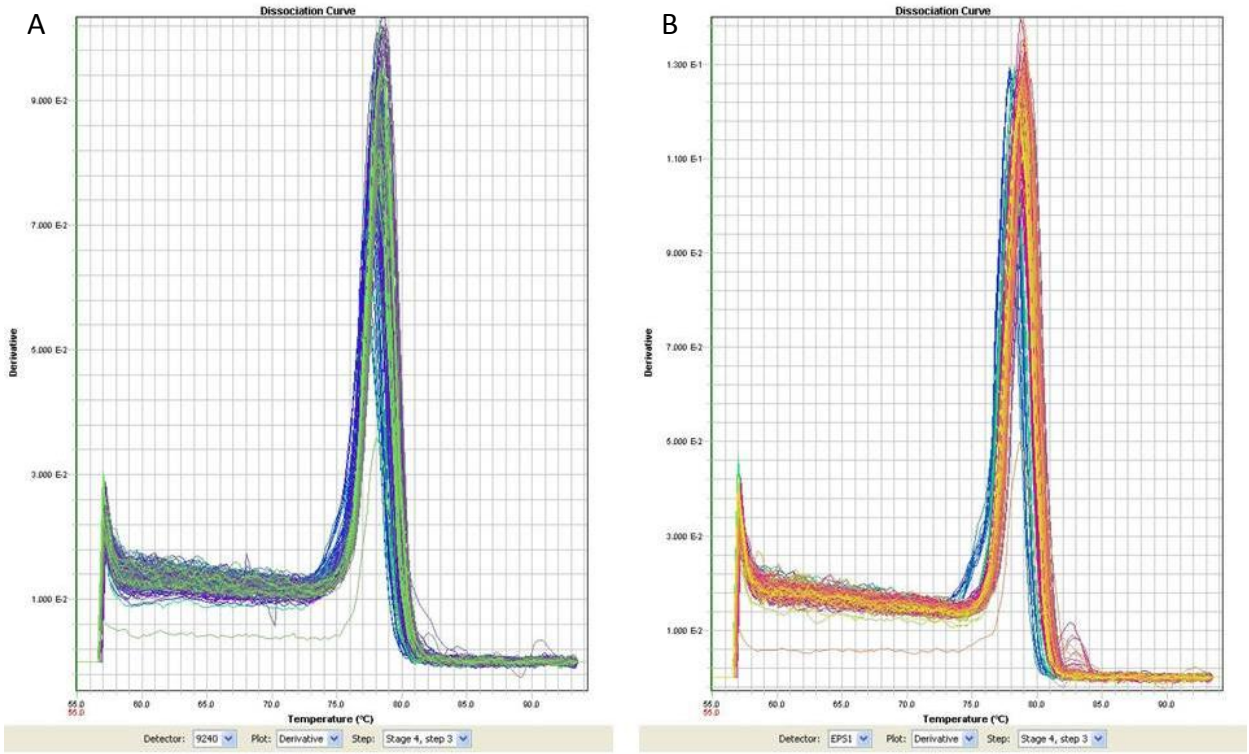
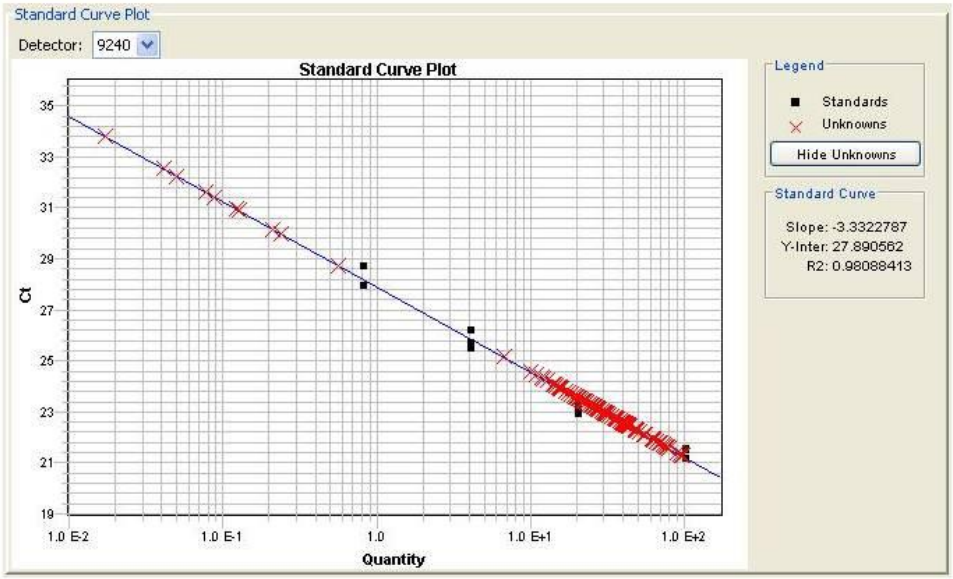


Figure 3.6 Examples of dissociation curves created in the final step of qPCR for 9240 (A) and *EPSPS* (B) amplifications. Curves were created by measuring fluorescence in qPCR samples. After PCR, the temperature was held at 95 C for 4 min to denature all double-stranded DNA. The temperature was then decreased to 55 C and held for 4 min to allow dye to intercalate into double stranded DNA molecules, in which state the dye becomes fluorescent. The temperature was then slowly ramped to 95 C, causing the denaturation of double-stranded products, beginning with the smallest such as possible primer dimers. During denaturation, dye molecules were released from the denatured products and discontinued fluorescing. Thus the presence of undesired products were deduced by the detection of any drop in fluorescence in the samples at temperatures other than those at which the desired products were known to become denatured (78 C). In this example, all products look generally clean, although in the *EPSPS* dissociation curve there is some evidence of possible products longer than the desired length, indicated by the short peaks occurring at approximately 82.5 C.

A



B

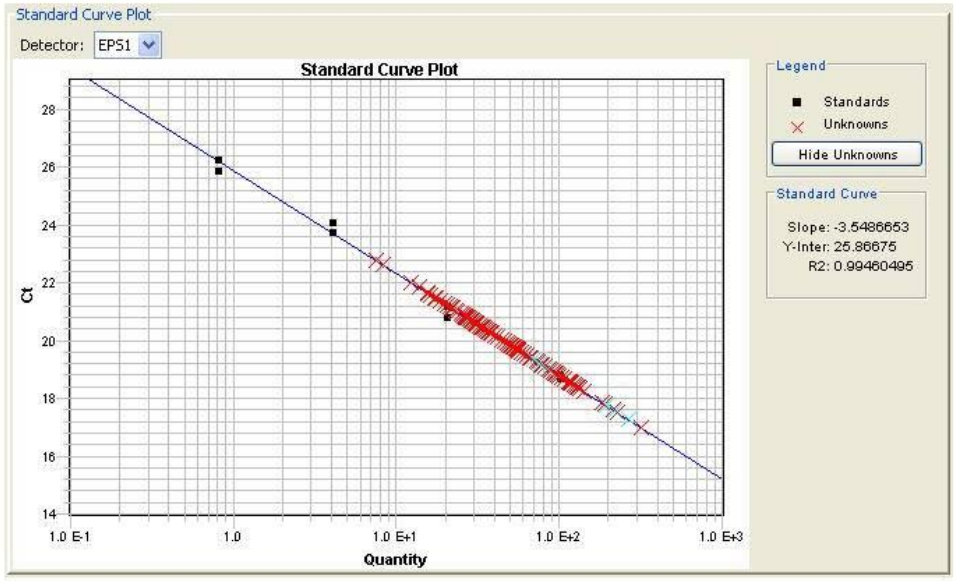


Figure 3.7 Example standard curve plots for 9240 (A) and *EPSPS* (B) demonstrating efficiency of PCRs at various concentrations of template DNA. The plots show C_t values (the number of cycles required for the PCR product to become detectable over the background in fluorescence readings) versus template concentration. Ideally the slope should be -3.32, which would indicate doubling of the product during each cycle in the PCR. Slopes between -3.6 and -3.1 fall within the acceptable range (90–110% efficiency). Correlation coefficients are considered acceptable when $R^2 \geq 0.97$.

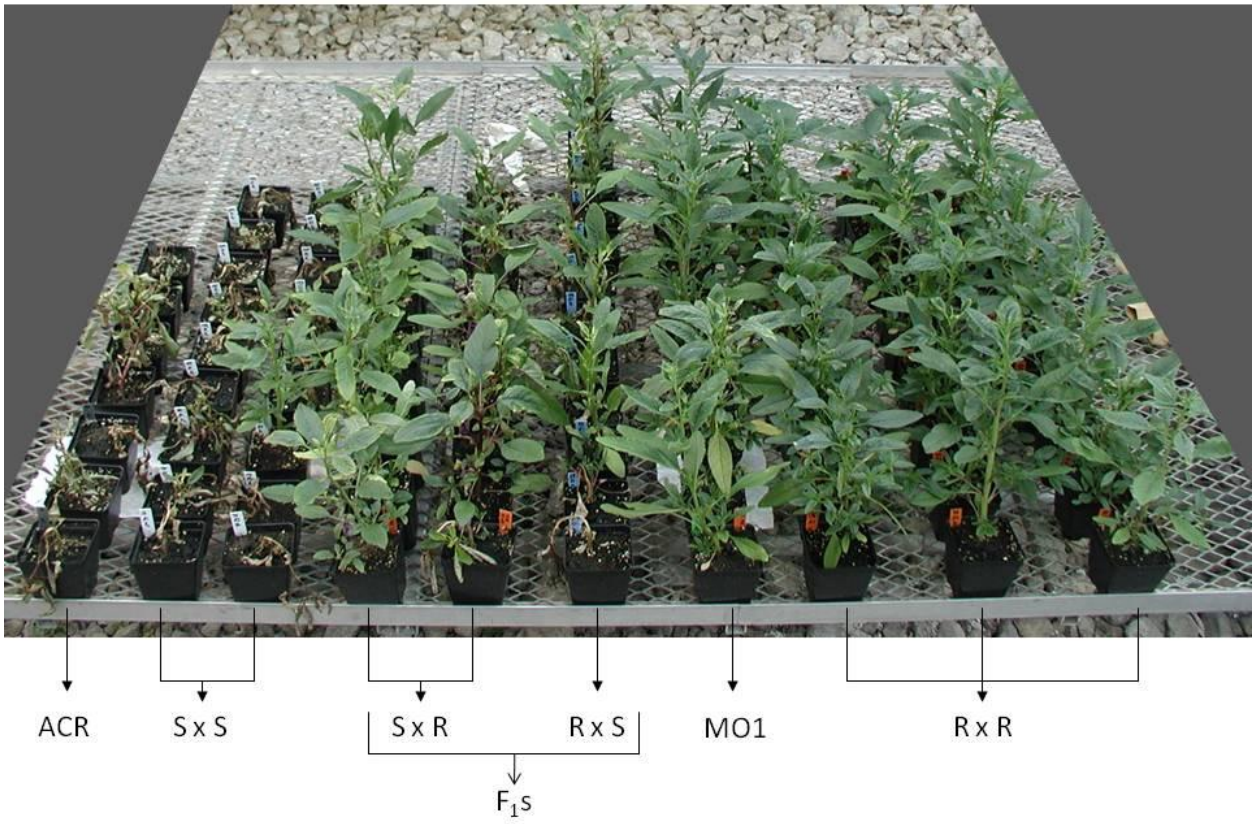


Figure 3.8 Photograph of the responses of F_1 plants to glyphosate at 420 g ae ha^{-1} in the initial screen. Plants shown from left to right are ACR, two rows of ACR x ACR test cross progeny (S x S), two rows of ACR x MO1 F_1 progeny (S x R) from two different families, MO1 x ACR F_1 progeny (R x S), MO1, and three rows of progeny from three different MO1 x MO1 crosses (R x R). Two of the ACR x ACR progeny were not controlled by glyphosate, which may indicate some pollen contamination. The leftmost F_1 line shows less segregation than the other two lines, and plants from this line were used to create F_2s and BC_S lines. The MO1 x MO1 progeny appear uniformly resistant to glyphosate, with phenotypes similar to those displayed by the parental MO1 line.

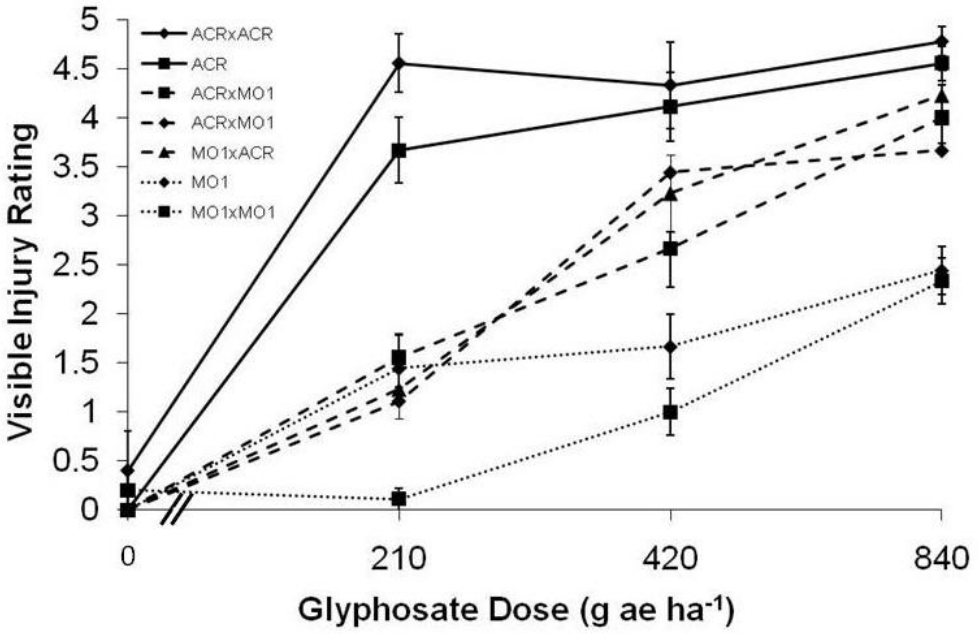


Figure 3.9 Visible injury ratings of test cross (ACR x ACR) progeny, ACR, F_1 progeny (dashed lines, ACR x MO1 and MO1 x ACR), MO1, and MO1 x MO1 progeny at 3 different rates of glyphosate. A rating of 0 corresponds to no injury to a plant, while a rating of 5 indicates a complete kill. F_1s generally display an intermediate level of injury between that of susceptible and resistant populations. This is due in part to segregation in the F_1 lines. The response to glyphosate is independent of the direction of the cross indicating nuclear inheritance of resistance.

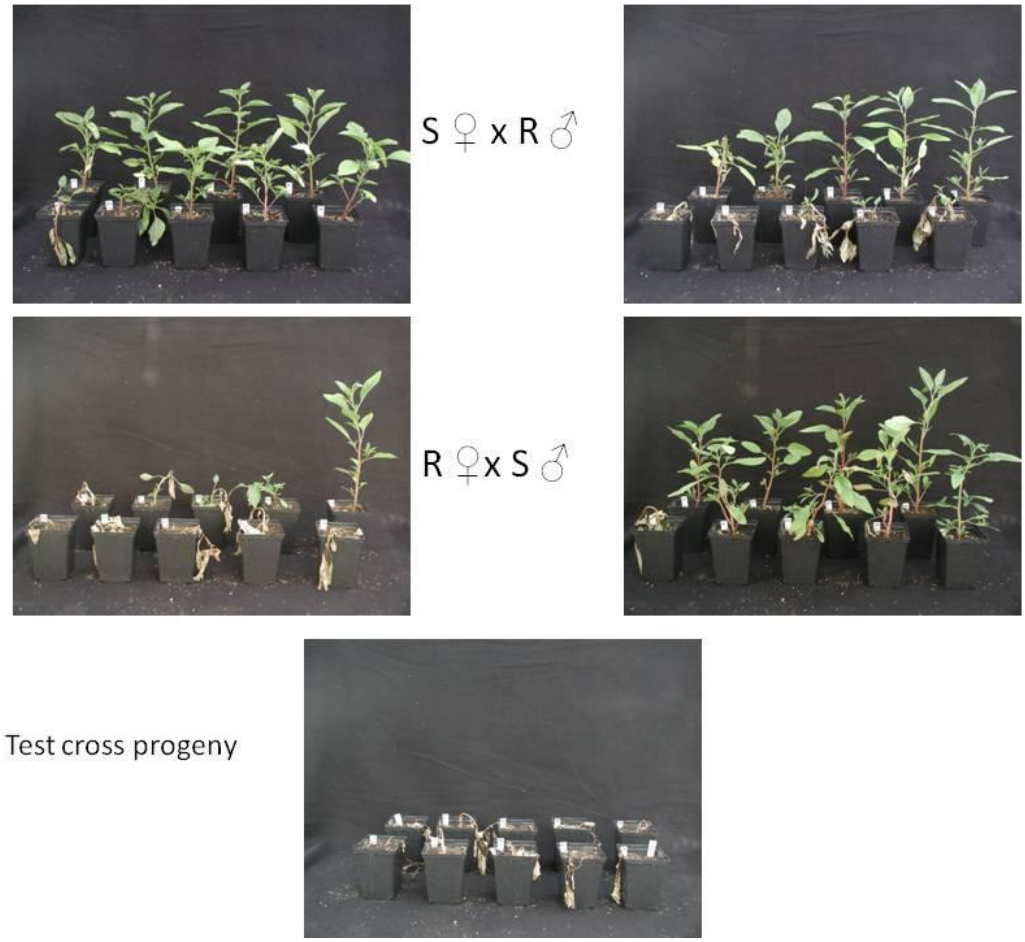


Figure 3.10 Photographs showing segregation for glyphosate resistance in the F_1 lines created during the cloning experiment. Plants were treated with glyphosate at 210 g ae ha^{-1}. Ratios ranged from nearly all resistant or intermediate to nearly all susceptible. These pictures also indicate that glyphosate resistance is nuclear inherited, as some plants survived regardless of the direction of the cross. Test cross progeny showed uniform susceptibility to glyphosate.

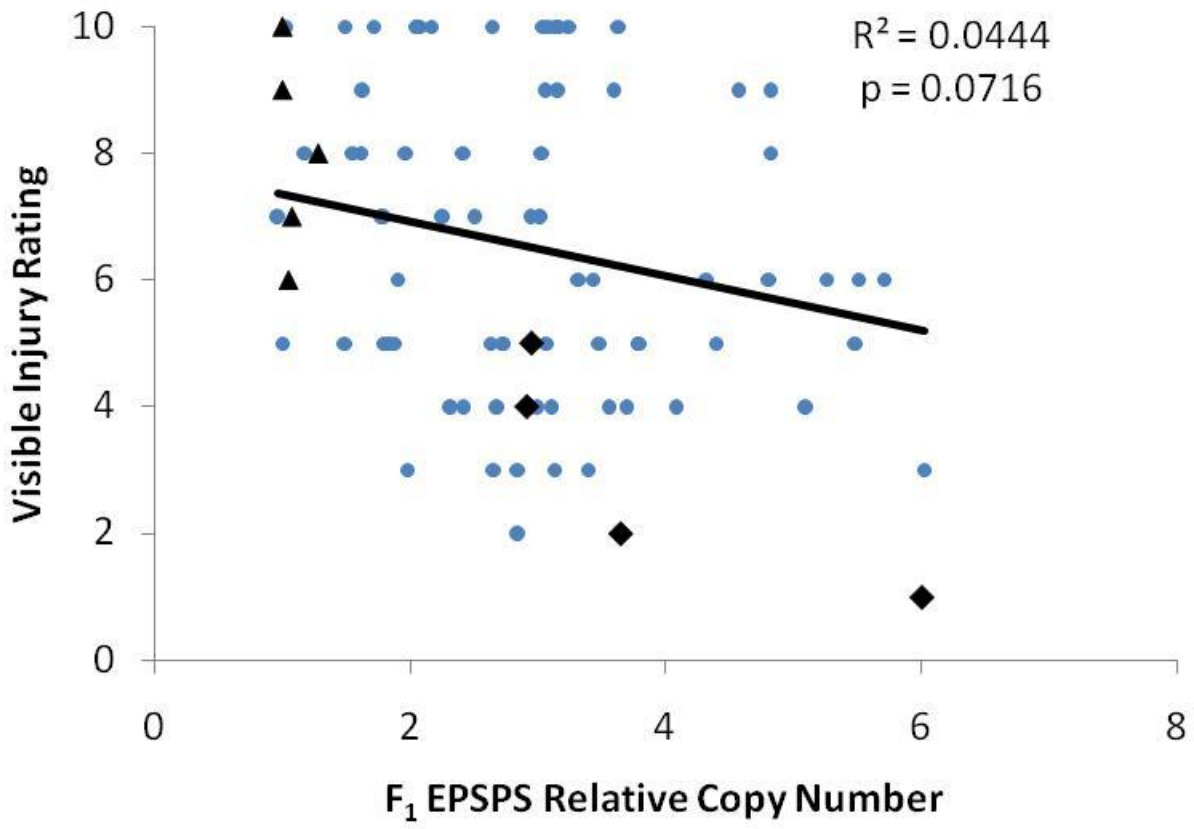


Figure 3.11 Visible injury rating of F_1 plants after glyphosate application at 1260 g ae ha^{-1} versus relative copy number of *EPSPS* compared with a susceptible control plant. A rating of 0 corresponds to no injury, and a rating of 10 corresponds to a complete kill. ACR susceptible control plants (▲) all showed relative copy numbers of nearly 1, with injury ratings of 6 or higher. MO1 resistant control plants (◆) showed increased copy number and lower injury levels, with ratings of 5 or less. F_1 plants (●) showed a range of relative copy number and injury ratings. The trendline suggests a potential weak correlation between resistance level and increased relative copy number, although the p-value indicates that this relationship is insignificant at the 0.05 confidence level.

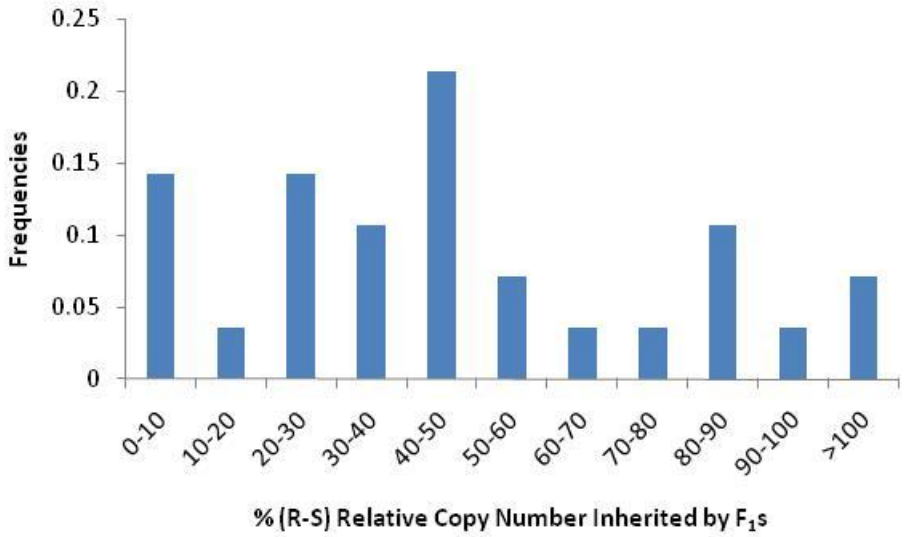


Figure 3.12 Inherited relative copy number of F_1 plants from one run of qPCR. Data represent 28 plants from 5 different families. Due to differing levels of relative copy number in F_1 parents, inherited relative copy numbers of F_1 plants from each family were normalized to the difference in copy number between the parents of each corresponding family. This was done by subtracting the relative copy number of the susceptible parent from that of the F_1s of the same family and dividing this difference by the difference between the R and S parents of the corresponding F_1 family. In this way, F_1 plants from all families may be shown on the same chart. This figure demonstrates that relative copy number is not inherited as a single gene trait, perhaps indicating that copies of *EPSPS* are located in multiple regions of the genome of MO1.

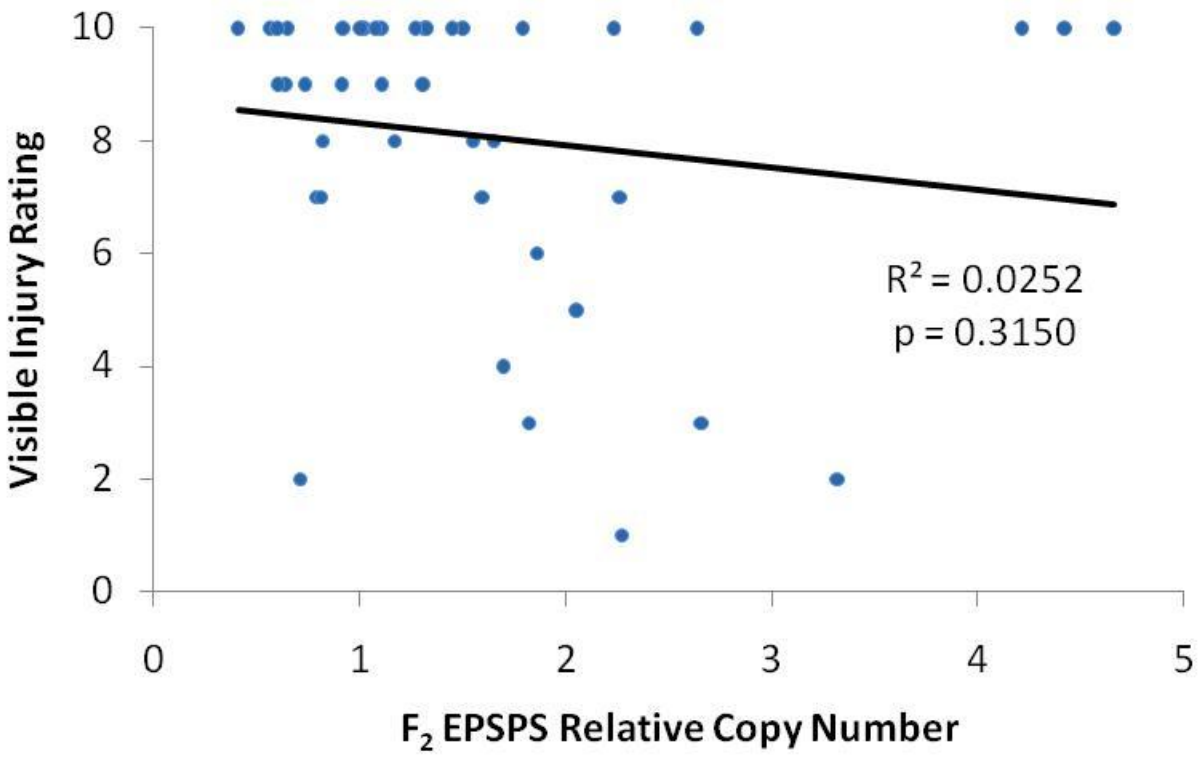


Figure 3.13 Visible injury rating versus relative copy number of *EPSPS* for F_2s receiving glyphosate treatment at 1680 g ae ha^{-1} (2x). A rating of 0 corresponds to an uninjured plant, and a rating of 10 corresponds to a complete kill. Relative copy number was compared with that of a susceptible control. The figure shows F_2 plants possessing a range of copy number from less than that of the susceptible control (normalized to 1) up to nearly five times the number of copies of the susceptible control. Diagram shows plants with low injury at a range of copy numbers, although the trendline again suggests a very weak correlation between relative copy number and resistance level, although the p-value of 0.3150 indicates that this relationship is in fact insignificant.

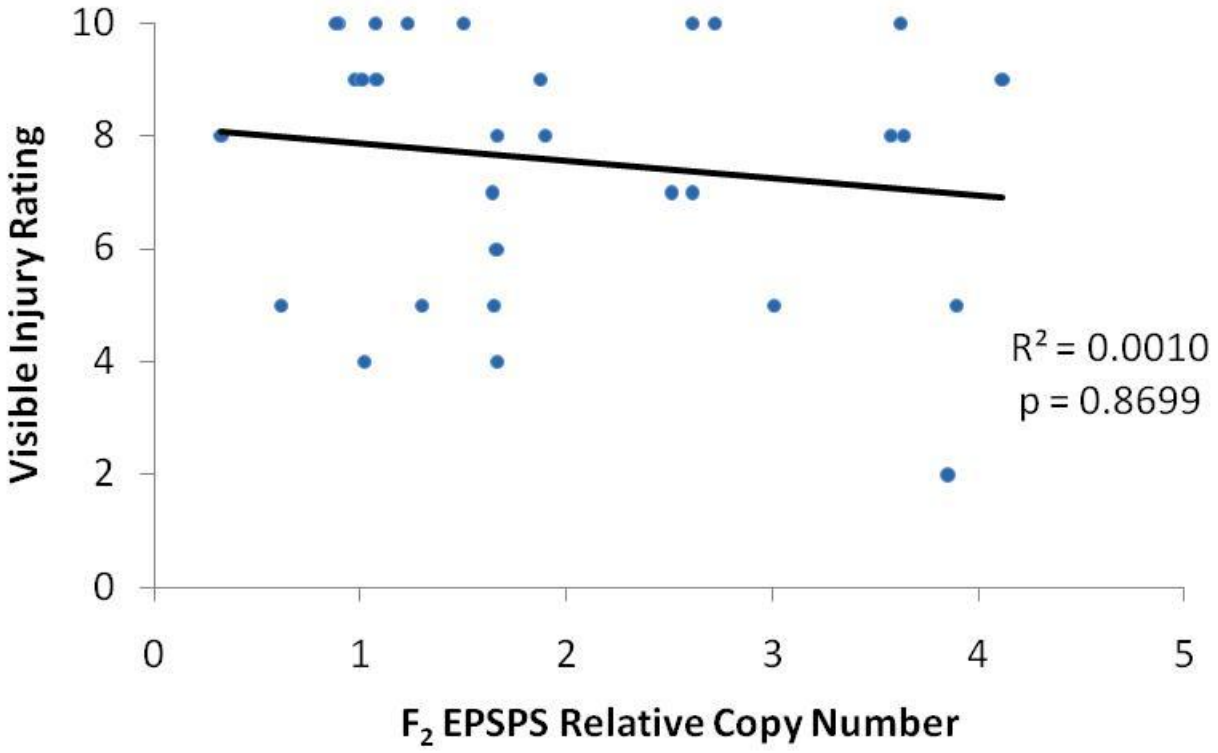


Figure 3.14 Visible injury rating versus relative copy number of *EPSPS* for F_2s receiving glyphosate treatment at 4200 g ae ha^{-1} (5x). A rating of 0 corresponds to an uninjured plant, and a rating of 10 corresponds to a complete kill. Relative copy number was compared with that of a susceptible control. The figure shows F_2 plants possessing a range of copy number from less than that of the susceptible control (normalized to 1) up to slightly more than four times the number of copies of the susceptible control. Diagram shows plants with low injury at a range of copy numbers, although the trendline again suggests a very weak correlation between relative copy number and resistance level, but the p-value of 0.8699 indicates that the non-zero slope of the line is likely due to chance, rather than a true significant relationship.

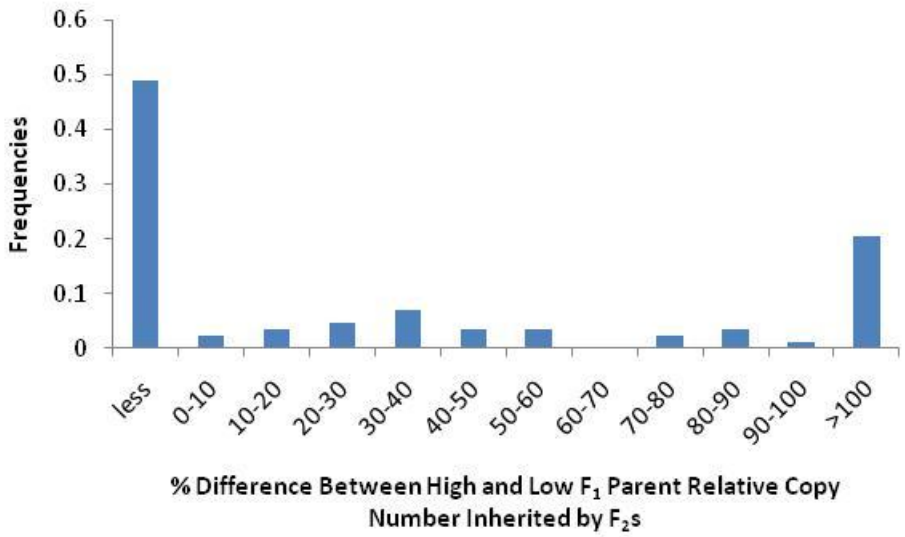


Figure 3.15 Inherited relative copy number of 88 F_2 plants in five families. Relative copy number in the F_2s was normalized to the difference between those of the parental F_1s by calculating the difference in relative copy number between an F_2 plant and the F_1 parent with lowest copy number. This difference was divided by the difference between the F_1 plant with high copy number and the F_1 plant with low copy number. Thus, an F_2 plant with 50% inherited relative copy number difference would have a relative copy number half-way between those of its parents. This figure again suggests that copy number is inherited as a quantitative trait, as it shows transgressive segregation, with nearly 70% of the F_2 plants containing relative copy number outside of the range of the parents.

Table 3.1 Segregation for glyphosate resistance in BC_S lines. BC_S lines passed a χ^2 test for homogeneity at both doses and thus the 5 lines screened were pooled at each dose. Expected ratios for BC_S lines were 1:1 (I:S) under the assumption of glyphosate resistance being a single partially dominant gene. At both glyphosate doses, the pooled BC_S lines fail a χ^2 test for 1:1 segregation.

		Populations						
					BC_S			
Rate	Phenotype	ACR	MO1	F_1	obs	exp	χ^2	p-value
	R	0	10	0	0	0		
1680	I	0	0	13	36	57.5		
	S	10	0	5	79	57.5	16.1	<0.0001
	R	0	9	0	0	0		
3360	I	0	0	10	21	57		
	S	9	0	8	93	57	45.5	<<0.0001

Table 3.2 Segregation for glyphosate resistance in BC_S lines with modified expectations. BC_S lines were pooled due to homogeneity among lines. Due to the death of F_1 plants expected to demonstrate an intermediate level of glyphosate resistance, expectations of BC_S segregation ratios were modified from the originally expected 1:1 segregation. 5 out of 18 (28%) of the supposed intermediate F_1 plants were controlled by glyphosate. Thus BC_S expectations were modified under the assumption that 28% of the expected intermediate plants would display a susceptible phenotype. This 28% was added to the number of plants originally expected to display a susceptible phenotype after glyphosate treatment. With modified expectations, the pooled BC_S lines pass the χ^2 single gene test at the low rate of glyphosate, although they still fail to pass at the high rate.

		Populations						
					BC_S			
Rate	Phenotype	ACR	MO1	F_1	obs	Exp	χ^2	p-value
	R	0	10	0	0	0		
1680	I	0	0	13	36	41.5		
	S	10	0	5	79	73.5	1.2	0.273
	R	0	9	0	0	0		
3360	I	0	0	10	21	31.7		
	S	9	0	8	93	82.3	5.0	0.025

Table 3.3 Segregation for glyphosate resistance in F_2 lines. Due to difficulty in differentiating between intermediate and susceptible plants in the BC_S analysis, F_2 lines were analyzed by grouping intermediate and susceptible phenotypes together into one phenotype (S). Thus the expected segregation ratios for an partially dominant single gene trait became 1:3 (R:S). At the low rate of glyphosate, all three of the F_2 lines pass a χ^2 test for a single gene trait. However, at the higher rate of glyphosate only one out of three of the F_2 lines passes the χ^2 test.

		Populations						
					F_2 observations			
Rate	Pheno.	ACR	MO1	F_1	MBX16	MBX18	MBX20	F_2 Exp.
	R	0	10	0	11	15	20	15
1680	I	0	0	13				
	S	10	0	5	49	45	40	45
				χ^2:	1.4	0	2.2	
				p:	0.233	1	0.136	
	R	0	9	0	8	8	18	15
3360	I	0	0	10				
	S	9	0	8	52	51	42	45
				χ^2:	4.4	4.1	0.8	
				p:	0.036	0.043	0.371	

Table 3.4 Segregation in clone F_1s. Out of 12 lines analyzed, all showed some level of segregation for glyphosate resistance.

Cross Type (female x male)	Phenotype	
	Intermediate	Susceptible
MO1 x ACR	8	1
	8	2
	7	3
	7	3
	6	4
	4	6
	3	7
	1	9
ACR x MO1	8	1
	7	2
	5	4
	4	6

CHAPTER 4

STACKING AND GENETIC LINKAGE OF FOUR HERBICIDE RESISTANCE TRAITS IN A SYNTHETIC WATERHEMP POPULATION

4.1 Abstract

The creation of a synthetic four-way resistant waterhemp population (a population containing four different types of herbicide resistances) that also contained individual plants resistant to all four types of herbicides is documented. Waterhemp plants from a glyphosate-resistant population were screened for resistance to glyphosate and then crossed with plants from a population resistant to acetolactate synthase (ALS)-inhibiting herbicides, protoporphyrinogen oxidase (PPO)-inhibiting herbicides, and triazines to produce F_1 seeds as part of a separate study on the inheritance of glyphosate resistance in waterhemp. The F_1 plants were again screened for glyphosate resistance and were crossed to produce F_2 seeds. Four different F_2 families, referred to as synthetic populations, were screened for resistance to all four herbicides. One such family, designated MBX5, which demonstrated segregation ratios closest to expected ratios of 3:1 for dominant single-gene traits, was selected for use in all subsequent experiments. The MBX5 population was then screened for four-way resistant individuals through sequential herbicide treatments as well as by the simultaneous application of atrazine, glyphosate, and lactofen following screening with a soil-applied treatment of imazethapyr. Numerous four-way resistant plants were identified in the population. Experiments were also performed to detect whether any of the resistance traits were linked. Initial resistance frequencies to each of the four herbicides separately were determined for the MBX5 population by screening plants with atrazine or glyphosate and by using molecular markers to detect ALS and PPO resistance within these same

plants. In the first run, the observed resistance frequencies were 66% for ALS, 65% for PPO, 82% for atrazine, and 85% for glyphosate, while in the second run the observed frequencies were 72%, 70%, 83%, and 38%, respectively. Next, plants determined to be resistant to one of these herbicides were tested for resistance to another of the herbicides, and the observed resistance ratios for the second herbicide were compared with the expected ratios in the absence of linkage. Chi-square tests were performed to detect significant deviations from expectations, and results indicated that ALS and PPO resistance traits were tightly linked with corresponding p-values much smaller than 0.001. Subsequent experiments involving the screening of MBX5 plants with ALS and PPO inhibiting herbicides rather than by using molecular markers confirmed that these two resistances were linked. This study shows that no major obstacles are present to prevent the combination of four types of herbicide resistance within a single waterhemp plant, indicating that four-way resistance is likely to evolve in the field. The finding that PPO resistance is closely linked with ALS resistance indicates that the two loci responsible for conferring resistance to these herbicides may be located close to one another on the same chromosome in the waterhemp genome.

4.2 Introduction

Waterhemp is a small-seeded summer annual plant indigenous to the Midwest United States (Sauer 1957), which has only recently become a major weed in agronomic cropping systems (Hager et al. 1997). This weed's relatively newfound success may be attributed to several factors. First, in recent years there has been a major shift toward no-till practices. Since waterhemp seeds are small, the seedlings only grow well when they germinate near the soil surface (Hager et al. 1997). Tillage tends to bury seeds in the soil, so lack of tillage allows more

of these seeds to germinate as compared with the number that would germinate in a field that has been tilled.

Waterhemp is a dioecious species, meaning that some plants are males that produce only pollen, while other plants are females that produce only seeds. The female plants are prolific seed producers. In fact some female waterhemp plants have been shown to produce in excess of 1×10^6 seeds during a growing season (Steckel et al. 2003). Waterhemp seeds may germinate throughout much of the growing season, making season-long control with a single post-emergence herbicide application difficult (Hager et al. 1997, Hartzler et al. 1999). It is often necessary for farmers to make multiple herbicide applications during the growing season to control this species, with the result of strong selection pressure for herbicide-resistant biotypes (Jasieniuk et al. 1996). These factors, when taken together, make this species a prime candidate for quickly evolving resistance to herbicides. In the past one and a half decades, waterhemp has evolved resistance to four herbicide modes of action (acetolactate synthase (ALS)-inhibitors, triazines, protoporphyrinogen oxidase (PPO)-inhibitors, and glyphosate) (Heap 2010), the majority of which are very important in controlling this species in soybean [*Glycine max* (L.) Merr.].

Waterhemp was first reported to be resistant to ALS inhibiting herbicides in 1993 (Heap 2010). Soon afterward, in 1994, triazine-resistant waterhemp was reported in Missouri. In 1996, the first multiple-resistant waterhemp population was reported, which was from Illinois and contained resistance to ALS inhibitors as well as triazines (Foes et al. 1998). In 2001 a waterhemp population was identified in Kansas, which contained individuals resistant to both PPO-inhibitors and ALS-inhibitors (Shoup et al. 2003). This report was followed a year later by

the report of a population in Illinois that demonstrated ALS-, PPO-, and PS II inhibitor resistance—the first 3-way resistant waterhemp population (Patzoldt et al. 2005).

Glyphosate resistance was identified in a Missouri waterhemp population in 2005 (Legleiter and Bradley 2008), marking the fourth type of herbicide resistance reported in waterhemp. This raised the question as to how difficult it would be to combine all four of these resistances into a single plant, as the answer to this question may provide some insight into the potential evolution and spread of four-way resistant waterhemp populations in the field. The objectives of the current study were to create a waterhemp population containing four-way resistant individuals in the greenhouse, as well as to investigate potential genetic linkage among the four types of herbicide resistance.

4.3 Materials and Methods

4.3.1 Greenhouse plant culture

All plants used for this study were grown from seed in the greenhouse. Seeds were sown in 12 cm x 12 cm x 5 cm containers in a medium consisting of a 3:1:1:1 mixture of commercial potting mix[1] to soil to peat to sand. When the seedlings exhibited two true leaves, they were transplanted into individual 6 cm x 4 cm x 5 cm inserts in 24 cm x 48 cm x 5 cm flats containing the previously mentioned growth medium. When plants reached 5 cm in height they were transplanted to 12 cm square pots containing 700 ml of growth medium, where they were allowed to grow until completion of the experiment. Plants were fertilized as needed using a slow-release complete fertilizer[2], and the plants were grown in the greenhouse under mercury halide and sodium vapor lamps that provided a minimum photon flux of 800 µmol m^{-2} s^{-1} at the plant canopy in addition to the light incident from the sun. The lamps were programmed for a 16-

h photoperiod, and the greenhouse was maintained at temperatures of 22 C at night and 28 C during the day. All plants were grown in this way unless otherwise indicated.

4.3.2 Herbicide application

All herbicide applications for this study were made using a compressed air, moving nozzle spray chamber with an adjustable platform and equipped with an 80015EVS even flat spray nozzle[3]. The nozzle was maintained at approximately 45 cm above the plant canopy. The sprayer was calibrated to deliver 187 L ha^{-1} of water at 207 kPa. Unless otherwise indicated, plants were sprayed when they reached 10–15 cm in height. Plants were returned to the greenhouse immediately after spraying.

4.3.3 Creation of synthetic population

While studying the inheritance of glyphosate resistance in a Missouri waterhemp population (Chapter 3) an attempt was made to create a synthetic population containing resistance to four herbicide modes of action. A glyphosate-resistant population designated as MO1, which was described previously (Legleiter and Bradley 2008), was screened with glyphosate[4] at rates of 420 – 3360 g ae ha^{-1} to identify glyphosate-resistant individuals. The spray solution also contained 2.5% (v/v) ammonium sulfate[5] (AMS). The plants were grown as described above and were treated with glyphosate when they reached 10–15 cm in height. The response of the MO1 plants to glyphosate was compared with that of the susceptible control, designated as Adams County Resistant (ACR), which was also previously described (Patzoldt et al. 2005). ACR, while susceptible to glyphosate, demonstrates uniform resistance to triazines, ALS-inhibitors, and PPO-inhibitors. Glyphosate-resistant MO1 plants were thus identified by

comparison with treated ACR plants and were subsequently allowed to grow until they began flowering, at which time they were crossed with untreated ACR plants to create F_1 seeds.

Crosses were performed by enclosing a single male plant (either MO1 or ACR) in a pollination bag[6] along with multiple female plants from both populations (Figure 4.1). Susceptible female plants were included in crosses containing a susceptible male as a test for pollen contamination. It was expected that if no foreign pollen entered the pollination bags enclosing the plants in such crosses, then the progeny collected from the susceptible females should all be susceptible to glyphosate. In order to maximize seed production, male plants were shaken daily to spread pollen throughout the enclosures, and females were kept with the males for about one month, or until the male plant in the tent appeared to have stopped producing viable pollen. Females were then harvested and allowed to dry at room temperature for at least two weeks, after which time seeds were manually harvested from each female. The seeds were then stratified to break dormancy. The stratification procedure consisted of the seeds first being surface sterilized by a 10 min treatment with a 1:1 bleach: water solution. Afterward, the seeds were washed twice with sterile deionized water, suspended in 0.15% (w/v) agarose, and then stored for at least two weeks at 4 C.

F_1 seeds were sown as described above. The emerging F_1 plants were screened with glyphosate at rates of 0, 210, 420, and 840 g ae ha^{-1} (including 2.5% (v/v) AMS) when they reached 10–15 cm in height to identify glyphosate-resistant individuals by comparison with the parental lines MO1 and ACR. Such individuals were then crossed through full-sib matings to create F_2 seeds using pollination bags as described above. Multiple F_1 females were included in crosses with each F_1 male (Figure 4.2), and the seed collected from an individual female plant made up an F_2 line, which was referred to as a synthetic population in this study.

4.3.4 Confirmation of four resistances in a synthetic population

Initially, four synthetic populations were screened for the presence of all four types of herbicide resistance that were present in the initial parental lines (glyphosate, ALS, PPO, and triazine resistance). Separate groups of 18 plants from each synthetic population were treated with each herbicide, and the results of the treatments were recorded at 14 days after treatment (DAT).

Treatments consisted of plants being sprayed with imazamox[7], atrazine[8], lactofen[9], or glyphosate[10]. Imazamox was applied at 44 g ae ha^{-1} and this treatment included 1% (v/v) crop oil concentrate[11] (COC) and 2.5% (v/v) AMS. Atrazine was applied at 1000 g ai ha^{-1} and included 1% (v/v) COC. Lactofen was applied at 110 g ai ha^{-1} and the treatment included 1% (v/v) COC and 0.25% (v/v) nonionic surfactant[12] (NIS). Glyphosate was applied at 630 g ae ha^{-1} and at 1260 g ae ha^{-1} and both treatments included 2.5% (v/v) AMS and 0.25% (v/v) NIS.

ACR was used as the resistant control for atrazine, imazamox, and lactofen, while MO1 was used as the resistant control for glyphosate. A population designated as Wayne County Susceptible (WCS), which was previously described (Patzoldt et al. 2005), was used as the susceptible control for all herbicides. Eight plants were used from each of the control biotypes for each treatment. The responses of plants from the synthetic populations were compared with those of the resistant and susceptible controls at 14 DAT and plants were given a rating of resistant (R), or susceptible (S). The experiment was performed as a completely randomized design (CRD). Following this experiment, one synthetic line, designated MBX5, was selected for use in all remaining synthetic-population experiments conducted in this study.

4.3.5 Screen for four-way resistant individuals

4.3.5.1 Sequential screen

An attempt was made to identify four-way resistant individual plants in the synthetic population. A concern during this experiment, however, was the possibility of antagonism among the herbicides, in which the presence of one herbicide in the mixture may directly or indirectly inhibit the herbicidal effect of another in the mixture (Green 1989). Thus, the four-way resistance screen was performed by applying the herbicides sequentially rather than simultaneously, in order to largely avoid potential antagonistic effects.

Seeds were sown as described above. One day after planting, the containers in which the seeds were sown received a soil-applied treatment of imazethapyr[13] at 1400 g ai ha^{-1} (20 times the field use rate) to screen for ALS-resistant seedlings. This high rate was chosen judiciously, as in an earlier experiment (discussed below) imazethapyr applied at 210 g ai ha^{-1} (3 times the field use rate) proved ineffective, presumably due to high content of organic matter in the growth medium. Soil receiving imazethapyr applications was lightly watered within 30 min of the herbicide treatment and was then watered as needed with a watering can (usually once per day).

Approximately 500 MBX5 seeds were screened in this way and were compared with treated controls of WCS and ACR, each containing about 100 seeds. In addition, an untreated WCS control, also containing approximately 100 seeds, was included to compare germination of seeds in the untreated soil with that of seeds in the herbicide-treated soil. This treatment was necessary because WCS seeds tend to have higher levels of dormancy than seeds from the other lines, and so it was desirable to have a measure of the baseline germination rate of WCS seeds in the absence of herbicide to evaluate the efficacy of the soil-applied herbicide treatment. In other words, the untreated 100-seed WCS control was included to test whether any germination of

untreated seeds occurred. Without this treatment, if no emergence occurred in the herbicide-treated seeds, it would not have been clear whether the lack of emergence was due to successful control by the herbicide or simply due to high levels of dormancy in the WCS seeds.

MBX5 seeds surviving treatment with imazethapyr were then grown as described earlier, until they were transplanted into 12 cm 700 ml square pots when they had reached 5 cm in height. At least 65 such plants were transplanted in each run of the experiment. After transplanting, the plants were allowed two days to recover, and were then treated with atrazine at 1000 g ai ha^{-1}, containing 1% (v/v) COC. MBX5 plants were scored as R or S at 7 to 9 DAT by comparison with the responses of eight R and eight S control plants, which were ACR and WCS, respectively.

At least 48 triazine-resistant MBX5 plants in each run were then sprayed with a mixture of glyphosate[14] at 4200 g ae ha^{-1} and lactofen at 110 g ai ha^{-1}, and including 2.5% (v/v) AMS and 0.25% (v/v) NIS. WCS, ACR, and MO1 were used as controls and were treated with the mixture as well as with glyphosate alone and with lactofen alone. WCS was expected to be susceptible to glyphosate, lactofen, and the combination of these two herbicides (Table 4.1). ACR was expected to be resistant to lactofen, but susceptible to glyphosate as well as the glyphosate-lactofen mixture. MO1 was expected to be resistant to glyphosate, but susceptible to lactofen and the glyphosate-lactofen mixture. By comparing the responses of the MBX5 plants receiving the glyphosate-lactofen mixture with those of the control treatments, four-way resistant individuals from the synthetic population were identified.

4.3.5.2 Simultaneous application

In addition to this conservative approach to avoid antagonism among herbicides while attempting to identify four-way resistant individuals, a second approach, involving the application of three herbicides simultaneously was also employed to achieve the same goal. In this approach, MBX5 plants were still screened with a soil-applied treatment of imazethapyr, but this herbicide was applied at a lower rate of 210 g ai ha^{-1} in the first run and at the usual 1400 g ai ha^{-1} in the second run. As described earlier, the 210 g ai ha^{-1} rate failed to control WCS seedlings and thus did not effectively screen for ALS-resistant plants. Again, this was probably due to the high level of organic matter in the soil, mainly due to the commercial potting mix.

Due to the failure of the soil-applied imazethapyr treatment in the first run to control WCS seedlings, MBX5 plants in the first run were re-screened for ALS resistance by treatment with imazamox at 44 g ai ha^{-1} in a mixture which included 1% (v/v) COC and 2.5% (v/v) AMS at the 5 cm stage, two days after being transplanted into 12 cm square 700 ml pots. The surviving (ALS-resistant) plants were then grown as described earlier until they reached 10–15 cm, at which time they were screened for resistance to the other three herbicides. In the second run of the experiment, however, the 1400 g ai ha^{-1} dose of imazethapyr did effectively control the susceptible WCS population, indicating that MBX5 seedlings emerging after such treatment were ALS-resistant. Thus these plants were grown as indicated above until they reached 10–15 cm, at which time they too were screened for resistance to the remaining three herbicides. In both runs of the experiment, ALS-resistant plants that had reached 10–15 cm in height were treated with a combination of atrazine at 1000 g ai ha^{-1}, glyphosate at 4200 g ae ha^{-1}, and lactofen at 110 g ai ha^{-1}, in a solution which included 2.5% (v/v) AMS and 0.25% (v/v) NIS.

The controls used to test for the efficacy of each herbicide (or to rule out antagonism) were somewhat more complicated in this case. WCS, ACR and MO1 were again used as the control populations. Treatments were made to all three control populations using every possible combination of atrazine, glyphosate and lactofen. Namely, these treatments were atrazine alone, glyphosate alone, lactofen alone, an atrazine-glyphosate mixture, an atrazine-lactofen mixture, a glyphosate-lactofen mixture, and a mixture of atrazine, glyphosate, and lactofen, where all herbicide rates were the same as those used on the MBX5 plants. The expected responses of the control populations to each of the treatments are summarized in Table 4.1.

By investigating the results of each of the control treatments and comparing the responses of the MBX5 plants receiving the atrazine-glyphosate-lactofen treatment with those of the control populations at 14 DAT, four-way resistant plants were identified. Both types of four-way resistance screens were performed as a CRD and they were repeated.

4.3.6 Linkage experiments

Potential genetic linkages among the four types of herbicide resistance were investigated in the synthetic population. The strategy was to first obtain reliable estimates of the resistance frequencies in the MBX5 population to each of atrazine, lactofen, imazamox, and glyphosate. After these estimates were obtained, groups of MBX5 plants that were resistant to one of the herbicides (e.g. atrazine) were investigated for resistance or susceptibility to one of the other herbicides (e.g. glyphosate). If the percentage of plants resistant to an herbicide in one of these subgroups was significantly different than the previously determined percentage of plants resistant to that herbicide alone, then this would indicate potential genetic linkage between the two types of resistance.

Due to the current lack of molecular markers for glyphosate resistance and non-target-site triazine resistance in waterhemp, for the purposes of the linkage study the frequencies of resistance to these herbicides were determined by treating the plants with herbicide. In the first run of this experiment, out of 160 MBX5 plants grown, 98 were treated with atrazine at 1000 g ai ha^{-1} and 1% (v/v) COC at the 5 cm stage to again check the frequency of triazine-resistant individuals in the population in the absence of selection with any other herbicide. At 9 DAT, the atrazine-treated plants were rated as either R or S by comparison with ACR and WCS, and the survivors were then treated with glyphosate at 3400 g ae ha^{-1} in a solution containing 2.5% (v/v) AMS and 0.25% (v/v) NIS. This treatment was applied to determine the frequency of glyphosate resistance in confirmed triazine-resistant plants. Meanwhile, the remaining 62 MBX5 plants (not sprayed with atrazine) were treated with glyphosate at 1180 g ae ha^{-1} when they reached 10–15 cm in height in order to test for the frequency of glyphosate resistance in the population in the absence of selection with any other herbicide. Plants from both groups were assigned ratings of R or S at 14 DAT with glyphosate. The higher rate of glyphosate applied to plants treated with atrazine was chosen to negate the possibility of plants being less susceptible to glyphosate due to stress from the atrazine treatment.

This same experiment was repeated, but in the second run 90 MBX5 plants were grown. Sixty of these were sprayed with atrazine at 1000 g ai ha^{-1} at 5 cm in height followed by treatment with glyphosate at 3400 g ae ha^{-1}, and the remaining 30 plants were sprayed only with glyphosate at 1180 g ae ha^{-1} when they reached 10–15 cm in height. Plants were again given ratings of R or S at 14 DAT with glyphosate. For all treatments, plants from the ACR, WCS, and MO1 populations were used as resistant and susceptible controls, with WCS susceptible to both

atrazine and glyphosate, ACR resistant to atrazine but susceptible to glyphosate, and MO1 susceptible to atrazine but resistant to glyphosate (Table 4.1).

Molecular markers were used to test for ALS and PPO-resistance in each of the MBX5 plants described above. ALS and PPO resistances in the synthetic line were assumed to have been inherited from the ACR parental line, and thus the resistance mechanisms for both types of resistance were known. ALS resistance in the ACR population is conferred by a single nucleotide mutation which causes the substitution of leucine for tryptophan at amino acid position 574 (W574L) in ALS (Patzoldt et al. 2007), while PPO resistance in the ACR population is conferred by the deletion of a glycine codon at amino acid residue 210 (ΔG210) in *PPX2* (Patzoldt et al. 2006). In both runs of the linkage experiment described above, 100 mg samples of meristematic tissue were collected from each of the MBX5 plants before treatment as well as from several untreated WCS and ACR plants, and these tissue samples were stored at -80 C until needed.

4.3.6.1 Molecular marker test for ALS-resistance

Total genomic DNA was extracted from all MBX5 plants in runs one and two of the linkage experiment using a modified hexadecyltrimethyl-ammonium bromide (CTAB) protocol from Doyle and Doyle (1990). Extracted DNA was resuspended in 50 µL of TE buffer. The DNA was then quantified using a spectrophotometer[15] and diluted to 50–100 ng μL^{-1} using sterile deionized water. Region B of *ALS* was amplified via polymerase chain reaction (PCR) using the primers described by Foes et al. (1998). PCRs contained 50–100 ng DNA, 0.2 mM of each dNTP[16], 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and reverse primers[17], and 0.5 units of Taq polymerase[18] with a 1x concentration of supplied buffer in a final volume of 20 µL.

During PCR, the samples were subjected to an initial denaturation step of 95 C for 3 min followed by 35 cycles of denaturation at 95 C for 30 s, annealing at 56 C for 1 min, and extension at 72 C for 1 min 30 s. The samples were then subjected to a final extension step of 72 C for 5 min.

Results of the PCR were checked via gel electrophoresis by running 5 µL of each product in a 1.0% (w/v) agarose gel. Desired products were identified as bands on the gel corresponding to fragments 451 base pairs (bp) in length (Figure 4.3, Panel A). The remaining 15 µL of product in samples demonstrating successful amplification of *ALS* were subjected to restriction enzyme digestion with *MfeI* as in Foes et al. (1999). Digestion reactions consisted of 0.2 µL BSA[19], 0.3 µL *MfeI* [20] (10 units μL^{-1}), 2.0 µL of the supplied 10X NEB4 buffer, 2.5 µL sterile deionized water, and 15 µL PCR product from *ALS* amplification, making a total reaction volume of 20 µL. Digestion reactions were allowed to proceed for 4–6 h at 37 C, after which time the products were investigated via gel electrophoresis by running 10 µL of each product on a 1.5% (w/v) agarose gel at 80 V for approximately 2.5 h. Plants were rated as susceptible if the band corresponding to the amplified region of *ALS* was uncut by the restriction enzyme, and plants were rated as resistant if the PCR product was cut by the enzyme, resulting in either one shorter band or both a shorter and a longer band visible in the gel. One of these bands was due to a cut fragment of DNA, while the other, longer fragment (the original 451 bp fragment) was due either to incomplete digestion or to heterozygosity of the plant.

4.3.6.2 Molecular marker test for PPO-resistance

The same diluted DNA used with molecular markers to test for ALS resistance as described above was also used to test for PPO resistance. Allele-specific primers PPX2LR-F and

PPX2Lex10-R (forward: 5'-TGT TGC GGG TAC ATG TGG A-3', reverse: 5'-CTG GAA ATG TAT GGT GCA TC-3') were used to amplify *PPX2* from resistant plants (gene containing the ΔG210 mutation). PCRs consisted of 50–100 ng DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and reverse primers, and 0.5 units of Taq polymerase with a 1x concentration of supplied buffer in a final volume of 20 µL. Resistant *PPX2* alleles were amplified using touchdown PCR. This consisted of an initial denaturation step of 95 C for 2 min followed by 35 cycles of 1 min of denaturation at 95 C, 30 s of annealing, and 45 s of extension at 72 C. The annealing temperature was 65 C in the first cycle and was decreased by 1 C in each cycle until it reached 55 C. The annealing temperature was then kept at 55 C for all of the remaining cycles. Samples were subjected to a final extension step of 72 C for 4 min at the end of the program and were then held at 4 C until needed. Results of the PCR were checked via gel electrophoresis by running 10 µL of each product in a 1.0% (w/v) agarose gel. If a band was present at 583 bp, the plant was scored as PPO-resistant, and if no band was present, the plant was scored as susceptible (Figure 4.4).

4.3.6.3 Linkage Analysis

Frequencies of triazine-, glyphosate-, ALS-, and PPO-resistant individuals were calculated for the MBX5 population based on the results of the treatment with atrazine, the treatment with glyphosate alone, and the tests with molecular markers. Next, plants that were confirmed resistant to a given type of herbicide were checked for resistance or susceptibility to one of the other herbicides, and the observed resistance ratios were compared with the previously calculated resistance ratio for that herbicide alone (which would be the expected resistance ratio in the case of no linkage). If the observed R:S ratios were significantly different than expected,

the existence of genetic linkage between the resistance traits was inferred. Significant deviations from expectations were detected by using a single degree of freedom chi-square (χ^2) test, and p-values were determined. Traits were considered linked for p-values ≤ 0.05.

Here an example is helpful for clarification. If the MBX5 population contained 75% resistance to atrazine and 75% resistance to ALS inhibitors, then in the case of no linkage between these two types of resistance, in analyzing the triazine-resistant MBX5 plants one would expect 75% of these plants to be resistant to ALS inhibitors, while the other 25% of the triazine-resistant plants would be susceptible to ALS inhibitors. If, however, one found that 100% of the triazine-resistant plants were also resistant to ALS inhibitors, for example, this would probably indicate linkage between ALS and triazine resistance. A sample χ^2 test for the above situation is provided below.

Assuming 80 MBX5 plants were screened with atrazine, based on the hypothetical resistance ratios provided above, one would expect that 60 of these plants should survive the treatment. Then in these remaining 60 plants, if there were no linkage, the expectation would be that 45 of these plants are resistant to ALS inhibitors, while the remaining 15 plants are susceptible to ALS inhibitors. However, assuming, as written above, that 100% of the triazine-resistant plants are also ALS-resistant, the χ^2 test for linkage would be performed as follows.

$$\chi^2 = \sum_{i=1}^{2} \frac{(observed_i - predicted_i)^2}{predicted_i} \qquad [1]$$

where $i = 1$ in this case would refer to triazine-resistant plants that are also resistant to ALS inhibitors and $i = 2$ would refer to the triazine-resistant plants that are susceptible to ALS inhibitors. Using the numbers for atrazine and ALS resistance provided in the example above, the calculation to test for linkage between these two types of herbicide resistance would continue as follows.

$$\chi^2 = \frac{(observed\ ALS\ R - predicted\ \ ALS\ R)^2}{predicted\ \ ALS\ R} + \frac{(observed\ ALS\ S - predicted\ \ ALS\ S)^2}{predicted\ \ ALS\ S} \quad [2]$$

$$\chi^2 = \frac{(60-45)^2}{45} + \frac{(0-15)^2}{15} = 20; \quad p = 0.000008 \quad [3]$$

Thus, in this example, the conclusion would be that ALS resistance and triazine resistance are linked. This type of calculation was performed for all pair-wise comparisons of herbicide resistance in both runs.

4.3.6.4 Herbicide screening to test for ALS and PPO linkage

Based on results of the previously described linkage experiments, the decision was made to test for linkage between ALS and PPO resistance by screening MBX5 plants with herbicides rather than relying solely on molecular marker data. Thus, 192 MBX5 plants were grown as described above until they reached 10–15 cm in height, at which time 91 plants were sprayed with imazamox at 44 g ai ha^{-1} in a solution containing 1% (v/v) COC and 2.5% (v/v) AMS, and 96 plants were sprayed with lactofen at 110 g ai ha^{-1} in a solution containing 1% (v/v) COC. The remaining 5 MBX5 plants were kept as untreated controls. Plants from the WCS, ACR, and MO1 populations were used as resistant and susceptible controls for both treatments.

Plants from both treatments were rated as R or S and resistance ratios to each of the separate herbicides were calculated. Surviving plants then received treatment with a second herbicide. Plants rated as resistant to imazamox were treated with lactofen at 110 g ai ha^{-1} in a solution containing 1% (v/v) COC, and plants rated as resistant to lactofen were treated with imazamox at 440 g ai ha^{-1} (10 times the previous application rate) in a solution containing 1% (v/v) COC and 2.5% (v/v) AMS. As all of the remaining MBX5 seeds from the initial seed lot were planted in this experiment, imazamox was applied at a higher rate to ensure that susceptible

MBX5 plants would be killed even if they had become less sensitive to herbicides due to stress from the lactofen treatment (because this experiment could no longer be repeated due to the lack of seeds). After the second herbicide application, the observed resistance ratios for the second herbicide treatment were compared with the expected ratios based on the results of the first herbicide treatment, and a single degree of freedom χ^2 test was performed to test for significant deviations from expectations as described above. Traits were considered linked for $p \leq 0.05$.

4.4 Results and Discussion

4.4.1 Confirmation of four resistances in a synthetic population

Each of the four synthetic populations screened was found to contain triazine resistance, ALS resistance, PPO resistance, and glyphosate resistance (Table 4.2). For triazine resistance, all four of the synthetic populations screened demonstrated segregation ratios close to the expected 3:1 for resistance conferred by a single dominant gene. As for PPO resistance, a known dominant, single-gene trait (Shoup et al. 2008), three of the four synthetic populations demonstrated approximately 50% resistance, while the MBX 16 line contained about 67% resistant individuals. This could potentially be explained by the fact that the F_1s were not screened for PPO resistance. Therefore, if the ACR parent used in the initial cross was heterozygous for PPO resistance (Rr ACR crossed with rr MO1), then the F_1s would have segregated 1:1 for PPO resistance. Further, since F_1s were not screened for PPO resistance, it would be possible that a heterozygous PPO-resistant F_1 (genotype Rr) was crossed with a PPO-susceptible F_1 (genotype rr). One would expect F_2s from such a cross to again segregate 1:1 for PPO resistance. The fact that the MBX 16 line demonstrated 67% resistant individuals could indicate that both F_1 parents used to create this line were heterozygous for PPO-resistance, since

the ratio 12:6 observed in this line easily passes a χ^2 test for a single gene trait with an expected 3:1 segregation ratio ($p = 0.41$).

The response of the synthetic populations to glyphosate was more difficult to quantify, as with glyphosate resistance, a broad range of phenotypes are usually displayed, from a phenotype equivalent to that of the resistant control to a phenotype equivalent to that of the susceptible control, and many phenotypes in between. Therefore, a cutoff response equivalent to that of the healthiest susceptible control plant was chosen and plants healthier than this were classified as R, while plants as healthy or less than the cutoff were classified as S. The table shows that three out of four of the synthetic populations (MBX 5, MBX 18, and MBX 20) contained at least two-thirds R individuals when treated with the lower rate of glyphosate, while at the higher rate, the frequency of resistant individuals dropped to roughly half that of the lower rate in MBX 5, MBX 16, and MBX 18. The frequency of resistant individuals at the higher rate of glyphosate was also lower in the MBX 20 line, but it did not drop as significantly as in the other three synthetic populations.

The response of the synthetic populations to the ALS inhibiting herbicide imazamox was rather interesting. ALS resistance is known to be a nuclear single-gene dominant or incompletely dominant trait (Tranel and Wright 2002; Boutsalis et al. 1999). However, all but one line (MBX5) showed R:S ratios much lower than the expected 3:1 ratio. In fact, in the MBX 16 population, only one plant out of 18 appeared to be resistant to this herbicide. Again, this effect may be explained, at least for the MBX20 population, by the fact that the F_1 plants were not screened for resistance to ALS inhibiting herbicides. Therefore, it is possible that ACR plants used as parents in the initial crosses were heterozygous for ALS resistance (Rr). If this were the case, then F_1 progeny from such plants (Rr ACR crossed with rr MO1) would be expected to

segregate 1:1 for resistance to ALS inhibiting herbicides with genotypes of Rr and rr. Since the F_1 plants were not screened for ALS resistance, it is possible that an F_1 plant heterozygous for ALS resistance (Rr) was crossed with an ALS-susceptible F_1 plant (rr). The resulting F_2 progeny from such a cross would again segregate 1:1 for ALS resistance, with genotypes of Rr and rr. Thus, this may have been the case for the MBX20 population, which demonstrated 39% ALS resistance, as the observed 7:11 segregation easily passes a χ^2 test for 1:1 segregation ($p = 0.35$). Why the resistance ratios of the MBX16 and MBX18 lines were so low is unknown.

Similar results of a failure of observed segregation ratios for a known single-gene trait to fit the expected segregation ratios for such a trait have been reported in other species. For instance, Tian et al. (2006) reported that segregation in an F_2 population of foxtail millet [*Setaria italica* (L.) Beauv.] for resistance to trifluralin, which is known to be a single-gene, recessive trait, failed to fit the expected 1:3 segregation. Rather than 25% resistance in the F_2s, the authors observed only 16.9% resistance, although this discrepancy between observation and expectation disappeared after further crosses and self-fertilization of the plants. This observation led the authors to conclude that possibly a modifier gene was linked with the α2-tubulin gene (the gene in which the presence of a mutation can confer resistance to trifluralin), causing the observed distortion in segregation. The authors suggest that this linkage may have been broken by crossing over in later generations. Perhaps a similar mechanism, responsible for distorting the observed segregation ratios for known single-gene traits, is present in waterhemp. However, as the goal of this aspect of the study was to confirm the presence of all four types of resistance in synthetic populations and to choose a synthetic population demonstrating approximately 3:1 segregation for each type of herbicide resistance, the cause of any deviations from expectations was not investigated. As the MBX5 population appeared to show segregation closest to expectations

across the four types of resistance, this population was selected for use in all subsequent experiments in this study. It should also be noted that in the screening of the test cross progeny created during the crosses described above, 2 plants out of 18 were observed to survive treatment with glyphosate, which may indicate a low level of pollen contamination among simultaneous crosses. If this were the case, it may also at least partially explain the observed distortion in segregation ratios.

4.4.2 Four-way resistance screen in synthetic population

4.4.2.1 Sequential screen

WCS was effectively controlled by the soil-applied treatment of imazethapyr. Thus, all surviving MBX5 seedlings were considered to have ALS resistance. In the first run of the experiment, 92 such ALS-resistant MBX5 plants were transplanted to pots and treated with atrazine as described above. Of the 92 plants transplanted, 66 were confirmed to be triazine-resistant, while the remaining 26 plants were susceptible to atrazine. 61 of the 66 triazine-resistant plants were then treated with the glyphosate-lactofen mixture. Five of the initial 66 triazine-resistant plants were discarded because, although they survived treatment with atrazine, their growth was stunted and thus they were significantly smaller than the other 61 plants at the time of treatment with the glyphosate-lactofen mixture.

Before recording results of this final treatment, the treatments were evaluated for effectiveness based on the results of the control populations. WCS was controlled by all three treatments of glyphosate alone, lactofen alone, and the glyphosate-lactofen mixture. ACR survived lactofen, but was controlled by glyphosate, as well as the mixture of glyphosate and lactofen. MO1 survived glyphosate, but was controlled by lactofen and the glyphosate-lactofen

mixture, indicating that little or no antagonism occurred from applying glyphosate and lactofen in combination. Thus, MBX5 plants surviving treatment with the glyphosate-lactofen mixture were considered four-way resistant individuals (Figure 4.5). In the first run of the experiment, 19 out of 61 of the individuals screened with the glyphosate-lactofen combination were identified as being four-way resistant. This indicates that 21% of the total number of ALS-resistant individuals screened in this run of the experiment were resistant to the other three herbicides.

In the second run of the experiment, 55 ALS-resistant MBX5 plants were transplanted to pots and then treated with atrazine. Of the 55 individuals screened, 40 were resistant to atrazine, while 15 were susceptible. Two of the 40 resistant plants were thrown out, again due to stunting. The remaining 38 triazine-resistant plants were then screened with the glyphosate-lactofen mixture, and 6 of these plants were found to be resistant to all four herbicides. Thus, in the second run of the experiment, 11% of ALS-resistant individuals were also resistant to the other three herbicides.

The discrepancy between the percentages of four-way resistant individuals between runs one and two is most likely attributable to differences in response to glyphosate between the two runs. However, because the purpose of this part of the study was to attempt to identify four-way resistant individuals, the cause of the difference was not investigated further.

4.4.2.2 Simultaneous application

The simultaneous applications of atrazine, glyphosate, and lactofen also allowed for the identification of four-way resistant MBX5 individuals. The control treatments in the first run worked well overall (Figure 4.6), although it appeared as if there may have been some antagonism in the mixture of all three herbicides (Panel G), as some ACR plants were not

completely killed with this treatment. It is worth noting, however, that these pictures were not taken until 37 DAT, which gave severely injured (but not quite killed) ACR plants time to begin to recover. As can be seen in Panel H, even with regrowth, the ACR plants look much less healthy than the surviving MBX5 plants. Therefore, these MBX 5 plants were considered to be four-way resistant individuals. One such individual is depicted in Figure 4.7, which shows a four-way resistant MBX5 individual compared with WCS controlled by glyphosate alone, atrazine alone, lactofen alone, and the combination of these three herbicides.

After the plants were given ratings of R or S, the R MBX5 plants in this first run of the experiment were transferred to another greenhouse room isolated from other waterhemp plants and were allowed to intermate in an attempt to acquire uniform seed lines of four-way resistant individuals. These seed lines are listed in Appendix B. 82 four-way resistant individuals were identified in this run out of a total of 169 MBX5 plants treated with the combination of atrazine, glyphosate, and lactofen.

Results were similar in the second run of the experiment (Figure 4.8), with the exception of slightly better control of the known susceptible populations. Interestingly, it appears that lactofen had a higher level of activity on plants in this run of the experiment. One example of this can be seen in Panel A, which shows the results of the lactofen treatment in which one of the ACR plants (resistant to lactofen) was killed by this herbicide. Panel D shows the results of the atrazine-lactofen mixture. ACR is resistant to both of these herbicides, and although all ACR plants did survive this treatment, some injury is clearly visible on plants of this population. The other herbicide combinations were quite effective, including the combination of atrazine, lactofen and glyphosate (Panel G). Panel H again shows the results of simultaneous treatment with three herbicides, but unlike Panel G, which shows only ACR, WCS, and MO1 from left to

right, Panel H shows plants from these three populations as well as four-way resistant and susceptible MBX5 plants. Thus, four-way resistant plants from the synthetic population were again identified in this run of the experiment. However, presumably due to the higher level of activity of lactofen in this run, only 17 MBX5 plants out of the 57 ALS-resistant plants that were sprayed with the combination of atrazine, glyphosate, and lactofen were rated as four-way resistant individuals.

4.4.3 Linkage analysis

Due to a difference in the responses of MBX5 plants to treatment with glyphosate between runs one and two, the first two runs of the linkage experiment were analyzed separately. In the first run of the linkage experiment, 66% of MBX5 individuals were found to be resistant to ALS inhibitors, 65% to PPO inhibitors, 82% to atrazine, and 62% to glyphosate (Table 4.3), which is in close agreement with the resistance frequencies found in the initial screening of the synthetic populations. For all pair-wise comparisons of herbicide resistance linkage analyzed in this run, the plants scored as resistant to herbicide 1 (through either herbicide screens or molecular marker analysis) generally demonstrated segregation ratios quite close to expectations based on the calculated resistance frequencies in the absence of selection with any other herbicide. In fact, all tests for linkage other than that for linkage between ALS and PPO resistance gave χ^2 values of 1.8 or less; for a single degree of freedom, this value corresponds to p-values of 0.18 or greater. However, the case was quite different in the test for ALS and PPO linkage. In this test, out of 105 plants identified as ALS-resistant, 94 of these same plants were found to be PPO-resistant, while only 11 of the ALS-resistant plants were susceptible to PPO inhibitors. This was much different than the expectation in the case of no linkage, as analysis of

all MBX5 plants in run one using molecular markers indicated that 65% of MBX5 plants are resistant to PPO inhibitors. Thus, out of 105 ALS-resistant plants, in the case of no linkage, 68 of these should be PPO-resistant and the remaining 37 should be PPO-susceptible. This difference between observed R:S segregation for PPO resistance in the ALS-resistant plants (94:11) and the expected segregation (68:37) led to a large χ^2 value of 28.2, which corresponds to a p-value of much less than 0.001, indicating that ALS and PPO resistance are linked.

In the second run of the linkage experiment, 72% of MBX5 plants were observed to be resistant to ALS inhibitors, with 70% resistant to PPO inhibitors, and 83% resistant to atrazine (Table 4.4). These results are quite similar to those obtained in the first run of the experiment. However, the observed frequency of resistance to glyphosate dropped markedly in the second run of the experiment (at 38%) when compared with the frequency of glyphosate resistance observed in the first run (62%). The cause of this difference is unknown. A higher level of glyphosate injury on the resistant control (MO1) population in the second run compared with the first (data not shown) suggests the observed difference in glyphosate resistance frequencies in the MBX5 population in the second run of the experiment may be due to unidentified (and thus uncontrolled) environmental factors that caused increased activity of glyphosate on plants. Such between-run differences in responses to glyphosate have been observed in other experiments (see Chapter 5 and Appendix A for a discussion on this effect).

Pair-wise comparisons were again performed between each of the four resistance types in this run, giving the same results obtained in the first run. No significant linkage was detected between any resistance types except for ALS and PPO resistance. In fact, all pair-wise comparisons other than the one between ALS and PPO resistance produced χ^2 values of 0.17 and smaller, corresponding to p-values of 0.68 and higher. In one case of non-linkage, PPO-resistant

plants were found to segregate precisely as expected for atrazine resistance, giving a χ^2 value of 0 and a p-value of 1.

ALS and PPO resistances were again found to be significantly linked in this run of the experiment. Out of 63 ALS-resistant plants identified in this run, 60 of these were found to also be resistant to PPO inhibitors, leaving only 3 ALS-resistant plants that were susceptible to PPO inhibitors. Based on the observed resistance frequency of MBX5 plants to PPO inhibitors in run two in the absence of any other selection, it was expected that only 44 of these ALS-resistant plants should be resistant to PPO inhibitors and the remaining 19 susceptible. The large difference between observed and expected produced a χ^2 value of 19.3 with a corresponding p-value again much smaller than 0.001. The results of these two runs of the linkage experiment are summarized in Table 4.5, which shows p-values for each of the linkage tests for runs one and two.

Due to these results, which were based on resistance frequencies observed from the use of molecular markers rather than herbicide screening, a final linkage experiment was performed in which ALS and PPO inhibiting herbicides (imazamox and lactofen, respectively) were used in the place of molecular markers to obtain resistance ratios. The results of this experiment, however, served only to confirm what had already been observed in the previous runs with the use of molecular markers. These results are shown in Table 4.6. Of 69 MBX5 plants surviving treatment with imazamox, 67 also survived treatment with lactofen, while 2 died from this treatment. And of 65 plants surviving an initial treatment with lactofen, 64 of these plants also survived the follow-up treatment with imazamox, and only 1 of the 65 plants died from this treatment.

The results of this study indicate that there are no major obstacles to combining four types of herbicide resistance into a single waterhemp plant. That this was performed rather easily in the greenhouse indicates that four-way resistant waterhemp could soon become a major problem in agronomic cropping systems in the Midwest.

Another interesting finding of this study is that little if any antagonism occurs among at least three of the herbicides used in this study—namely atrazine, glyphosate, and lactofen—when they are applied simultaneously. Although it would be a rare situation where all three of these herbicides are applied simultaneously (lactofen is used primarily to control broadleaf weeds in soybean, but atrazine is not labeled for soybean, and is used in corn), other combinations of two of these herbicides may be of some use. For instance, if a farmer has a problem with one species of weed that is glyphosate-resistant, but not PPO-resistant, he could potentially spray a mixture of lactofen and glyphosate in his glyphosate-resistant soybean to control all weeds without having to worry about decreased activity of either of these herbicides. Although the 4200 g ae ha^{-1} rate of glyphosate used in the three-herbicide mixtures applied in this study was rather high (5 times the field use rate), the atrazine rate of 1000 g ai ha^{-1} is the same as the field use rate, and the 110 g ai ha^{-1} rate of lactofen used in this study is actually only half that applied in the field, and so by increasing the rate of lactofen used in the herbicide mixture even better control of some weeds could potentially be achieved.

The linkage findings were quite interesting. All available data suggest that ALS and PPO resistance are closely linked. A couple of possible explanations for this observation immediately come to mind. One explanation (which is incorrect) is that the same mechanism confers resistance to both types of herbicides. In other words, perhaps a single gene confers resistance to ALS and PPO inhibiting herbicides. If this were true, then in theory every plant resistant to ALS

inhibitors should also be resistant to PPO inhibitors, which is nearly what can be seen in the linkage data. However, this is known not to be the case. In fact, ALS resistance and PPO resistance are both known to be single-gene traits located at different loci. With this fact in mind, the data seem to suggest that these two loci may be located close to one another on the same chromosome. This hypothesis could be tested by attempting four different PCR reactions involving every possible combination of forward and reverse primers designed to amplify ALS and PPX2. For instance, one reaction would include a forward ALS primer with a forward PPX2 primer. Another reaction would include a forward ALS primer with a reverse PPX2 primer. Two other PCRs could be conducted using a reverse ALS primer with the forward and reverse PPX2 primers. Assuming that the primers are very specific and thus that they will only bind in one location within the genome, if a product was produced in one of these PCRs it could prove that the genes are closely linked. By determining the length of the PCR product, it would be possible to determine the distance between the two loci, which would be a very interesting project.

Herbicide resistance linkage has also been reported in rigid ryegrass [Lolium rigidum Gaudin] by Preston (2003). The author reported on linkage of metabolism-based cross-resistance to simazine (an inhibitor of photosystem II (PSII)), chlorotoluron (a PS II inhibitor), chlorsulfuron (an ALS inhibitor), and talkoxydim (an Acetyl-CoA carboxylase inhibitor), with the finding that two of these resistances—simazine resistance and chlorotoluron resistance—are linked.

The fact that PPO-resistance and ALS-resistance are linked in waterhemp should not be interpreted as meaning that they always occur together. To the contrary, populations have been found to contain resistance to only one or the other type of herbicide (Heap 2010). However, the fact that when both types of resistance are present in an individual plant they are often linked

may have implications for herbicide resistance management. In such cases of ALS- and PPO-resistant waterhemp populations, the resistance traits will travel together through pollen flow, and once-susceptible populations surrounding these fields may quickly evolve into populations containing a high proportion of multiple-resistant individual plants.

4.5 Sources of Materials

[1] LC1 professional growing mix, Sun Gro Horticulture Canada Ltd., 52130 RR 65, P.O. Box 189, Seba Beach, AB 70E 2BO Canada. Distributed by Sun Gro Horticulture Distribution Inc. 15831 N.E. 8th St., Suite 100, Bellevue, WA USA 98008.

[2] Scotts Osmocote Classic 13-13-13 slow-release fertilizer. The Scotts Company LLC, 14111 Scottslawn Rd., Marysville, OH 43041.

[3] TeeJet 80015EVS spray nozzle. TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187.

[4] Roundup WeatherMAX® Herbicide. Monsanto Company, St. Louis, MO 63167.

[5] N-Pak® AMS Liquid, Winfield Solutions, LLC, P.O. Box 64589, St. Paul, MN 55164-0589.

[6] 78" x 72" 1.75 mil Pollination Bags. Vilutis & Co., Inc. 1135 Center Rd., Frankfort, IL 60423.

[7] Raptor® herbicide. BASF Corporation. 26 Davis Drive, Research Triangle Park, NC 27709.

[8] Aatrex Nine-O. Syngenta International AG. Schwarzwaldallee 215 P.O. Box CH-4002, Basel, Switzerland.

[9] Cobra® herbicide. Valent U.S.A. Corporation. 1600 Riviera Ave., Suite 200, Walnut Creek, CA 94596-8025.

[10] Touchdown HiTech® Herbicide. Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419.

[11] Herbimax® Petroleum Oil-Surfactant Adjuvant. Loveland Products, Inc., P.O. Box 1286, Greeley, CO 80632.

[12] Activator 90 Nonionic Surfactant. Loveland Products, Inc. P.O. Box 1286, Greeley, CO 80632.

[13] Pursuit® Herbicide. BASF Corporation. 26 Davis Drive, Research Triangle Park, NC 27709.

[14] MON 76255 40.2% ae Technical Grade Glyphosate. Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167.

[15] Nanodrop 1000 Spectrophotometer v3.7.1. Thermo Fisher Scientific Inc., 81 Wyman St., Waltham, MA 02454.

[16] Invitrogen 100 mM dNTP Set, PCR Grade, Invitrogen Corporation, 5791 Van Allen Way, P.O. Box 6482, Carlsbad, CA 92008.

[17] IDT Custom Oligos. Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241.

[18] GoTaq Flexi DNA Polymerase. Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711.

[19] Purified BSA 100x. New England Biolabs, 240 County Road, Ipswich, MA 01938-2723.

[20] MfeI Restriction Enzyme. New England Biolabs, 240 County Road, Ipswich, MA 01938-2723.

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Tranel, P. J., and T. R. Wright. 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci. 50:700–712.

4.7 Tables and Figures

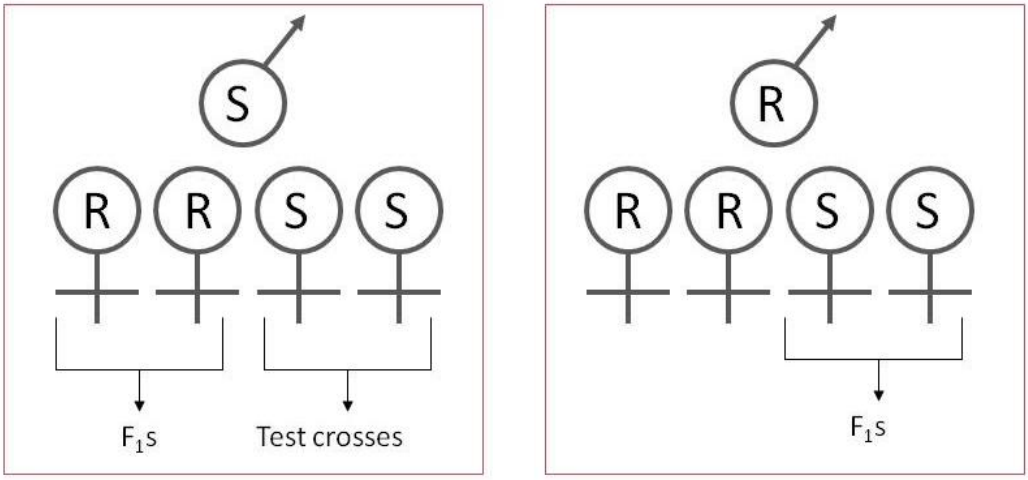

Figure 4.1 Diagrams of crosses utilized to create F_1 plants. Here S indicates ACR and R indicates MO1. Susceptible females were included in crosses with susceptible males as a test for pollen contamination (labeled as test crosses). Progeny from such crosses should be susceptible to glyphosate in the absence of contamination with R pollen.

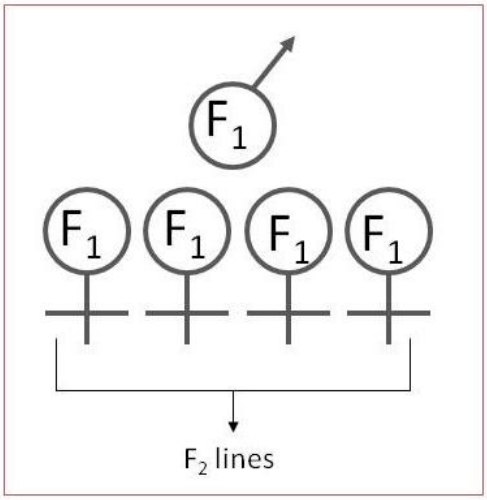

Figure 4.2 Diagram of crosses utilized to create F_2s. Seeds collected from an individual F_1 female constituted a synthetic F_2 population. All F_1 plants involved in a particular cross were grown from seed collected from a single female plant that was pollinated by a single male, and thus were full-sibs.

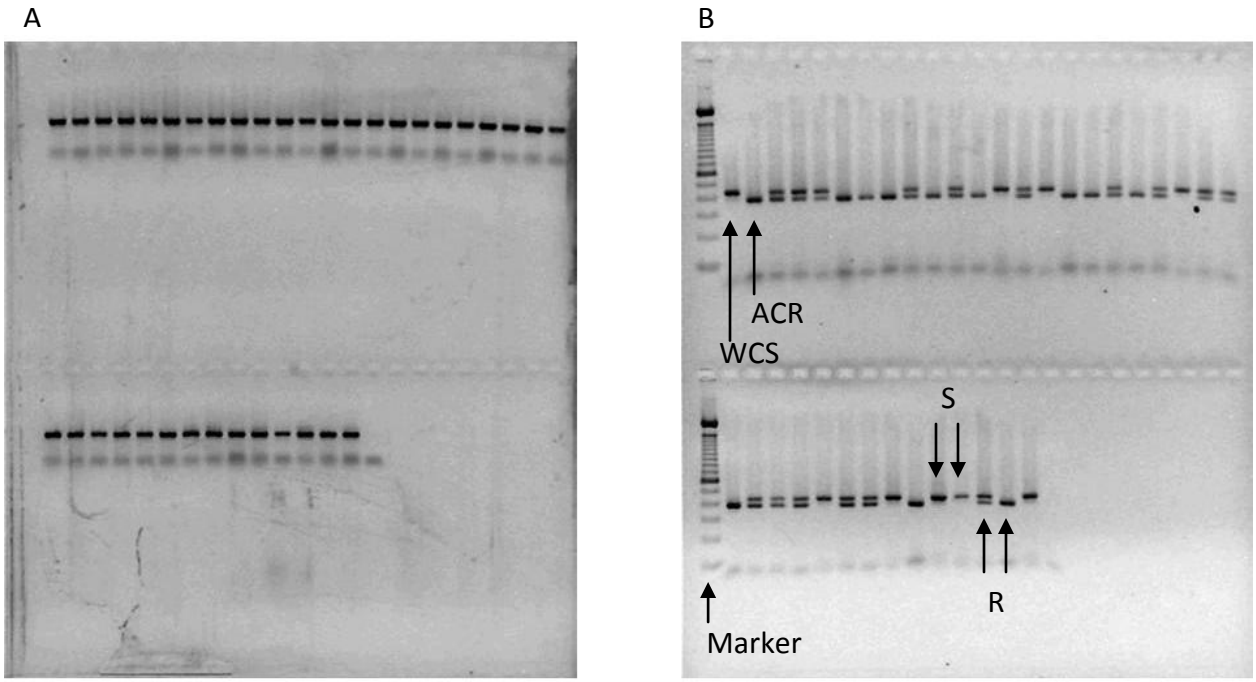

Figure 4.3 Example pictures of agarose gels showing successful amplification of *ALS* (A), and digestion of *ALS* products (B). Bands that were uncut by the restriction enzyme (like the susceptible control, WCS) indicate susceptible plants. Samples containing a shorter band due to being cut by the restriction enzyme (like the resistant control, ACR) are resistant to ALS inhibiting herbicides. Samples corresponding to R plants showing two bands after digestion indicate a heterozygous plant. Although in some instances the presence of two bands may indicate an incomplete digestion of the product, in this particular example, because the resistant ACR plant shows only one short band, other samples showing two bands in (B) must have been heterozygous plants.

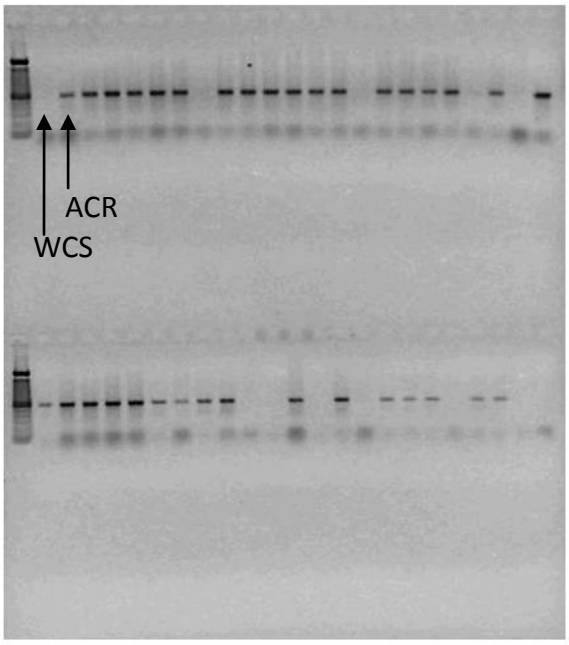

Figure 4.4 Example of molecular marker results for determination of MBX5 plants' resistance or susceptibility to PPO inhibitors. Allele-specific primers were used, which only amplified the resistant allele. WCS (the susceptible control) shows no band, indicating susceptibility to PPO-inhibiting herbicides. ACR (the resistant control) showed a band, indicating resistance to PPO inhibitors. Thus, all plants showing bands on the gel were identified as PPO-resistant individuals.

Figure 4.5 Results of the application of the glyphosate-lactofen mixture to plants in the first run of the four-way resistant individual screen using sequential herbicide applications. Plants shown from left to right are MBX5 plants resistant to ALS inhibitors and triazines, but susceptible to the combination of glyphosate and lactofen, ACR (which was controlled with the glyphosate in the mixture), MO1 (which was controlled by the lactofen in the mixture), WCS (which is susceptible to both glyphosate and lactofen), and four-way resistant MBX5 plants, surviving first a soil-applied treatment of imazethapyr at 1400 g ai ha^{-1} followed by (fb) a treatment with atrazine at 1000 g ai ha^{-1} fb treatment with a combination of glyphosate at 4200 g ae ha^{-1} and lactofen at 110 g ai ha^{-1}.

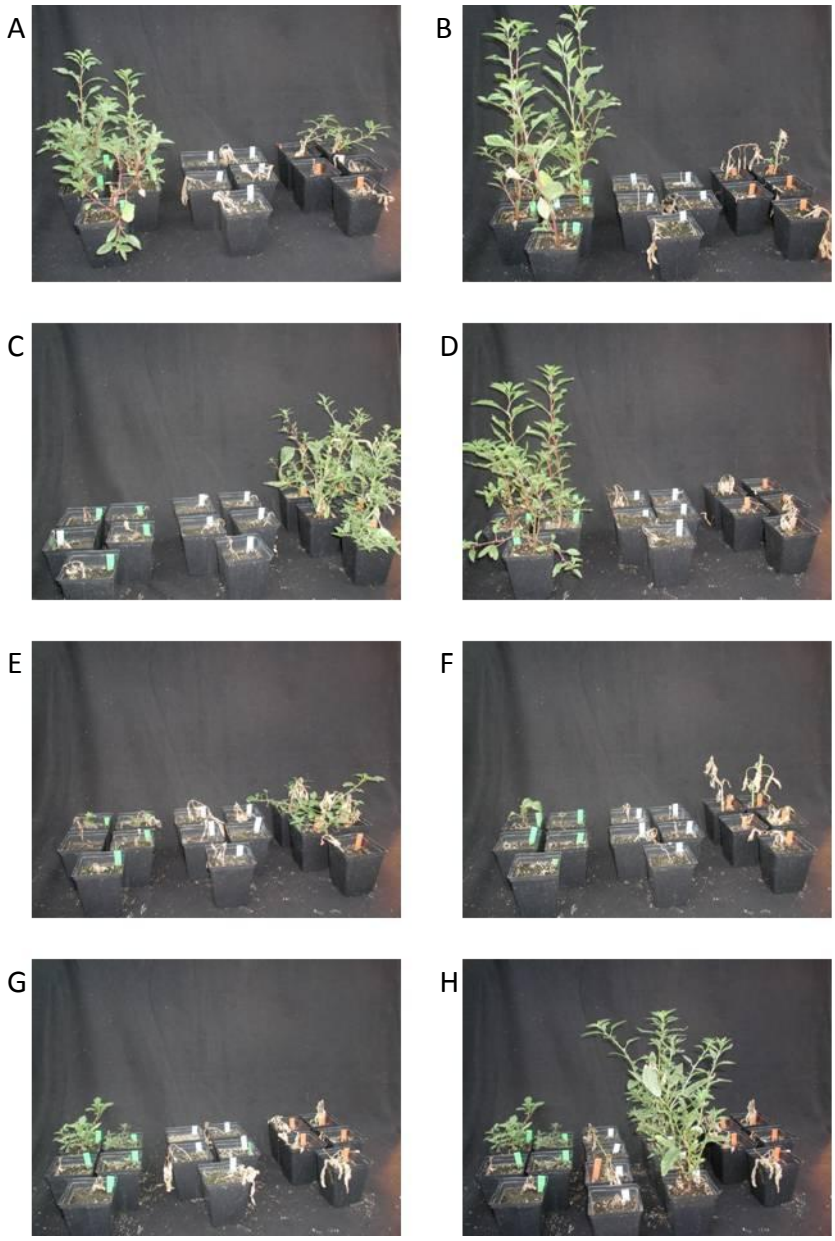

Figure 4.6 Results of the first run of the screen for four-way resistant individuals using a simultaneous application of atrazine, glyphosate, and lactofen. Pictures show the responses of plants of the control populations to lactofen (A), atrazine (B), glyphosate (C), lactofen and atrazine (D), lactofen and glyphosate (E), glyphosate and atrazine (F), and the combination of atrazine, glyphosate and lactofen (G, H). Plants shown from left to right are ACR, WCS and MO1 in A–G, and ACR, WCS, MBX5, and MO1 in H. Pictures were taken at 37 DAT.

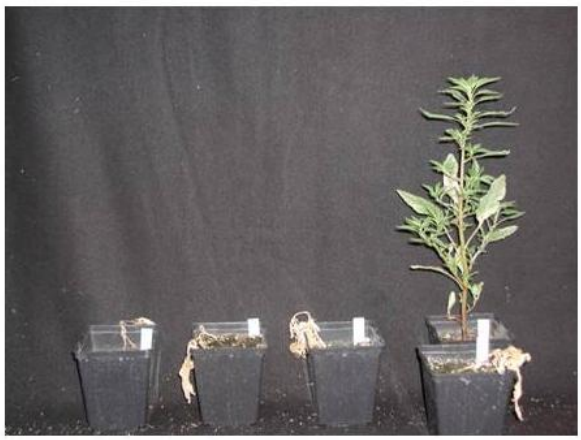

Figure 4.7 A four-way resistant MBX5 individual identified by screening MBX5 plants with a treatment of imazamox at 44 g ai ha^{-1} at the 5 cm stage followed by (fb) treatment with atrazine, glyphosate, and lactofen simultaneously. The other plants pictured from left to right are WCS controlled by glyphosate, WCS controlled by atrazine, WCS controlled by lactofen, and WCS controlled by a combination of all three of these herbicides (in the foreground).

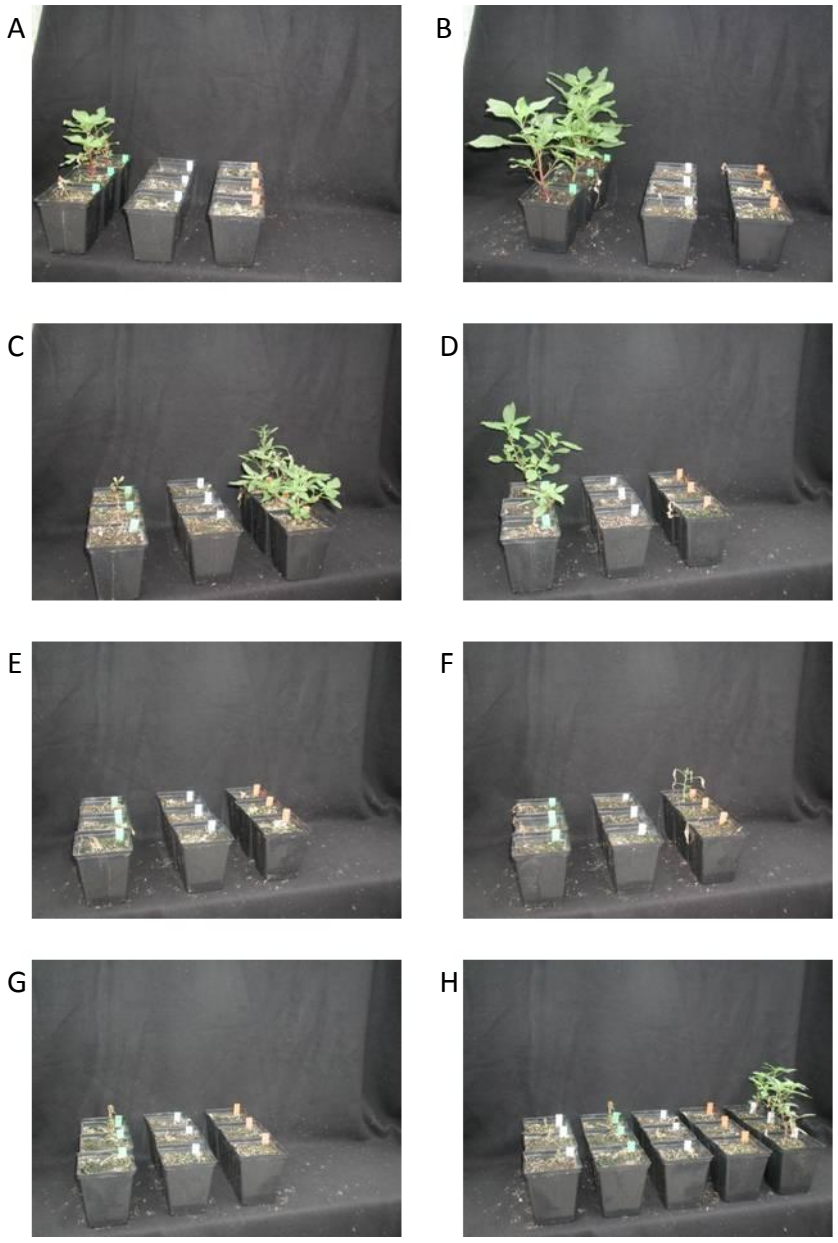

Figure 4.8 Results of the second run of the screen for four-way resistant individuals using a simultaneous application of atrazine, glyphosate, and lactofen. Pictures show the responses of plants of the control populations to lactofen (A), atrazine (B), glyphosate (C), lactofen and atrazine (D), lactofen and glyphosate (E), glyphosate and atrazine (F), and the combination of atrazine, glyphosate and lactofen (G, H). Plants shown from left to right are ACR, WCS and MO1 in A–G, and susceptible MBX5, ACR, WCS, MO1, and resistant MBX5 in H.

Table 4.1 Expected responses of susceptible and resistant control populations to three different herbicides and all combinations of the three, which were applied to test efficacy of the herbicides separately and in combination in several experiments conducted in this study in search of four-way resistant individuals. WCS is susceptible to all herbicides. ACR is resistant to atrazine and lactofen (as well as ALS inhibiting herbicides not applied in this mixture), but is susceptible to glyphosate. MO1 is susceptible to atrazine and lactofen, but is resistant to glyphosate. The responses indicated below are the expectations in the absence of antagonism among herbicides.

Treatments	Expected Responses		
	WCS	ACR	MO1
Atrazine	S	R	S
Lactofen	S	R	S
Glyphosate	S	S	R
Atrazine + Lactofen	S	R	S
Atrazine + Glyphosate	S	S	S
Lactofen + Glyphosate	S	S	S
Atrazine + Lactofen + Glyphosate	S	S	S

Table 4.2 Results of initial screen of synthetic (F_2) populations with multiple herbicides. Data shown are the number of resistant plants (R), the number of susceptible plants (S), and the percentage of resistant plants (% R) for each population and each herbicide treatment.

	Treatments[a]														
	Atrazine			lactofen			imazamox			Glyphosate 0.75x			Glyphosate 1.5x		
Line	R	S	% R[b]	R	S	% R	R	S	% R	R	S	% R	R	S	% R
MBX5	12	6	67 ± 11	10	8	56 ± 12	15	3	83 ± 9	12	6	67 ± 11	5	13	28 ± 11
MBX16	14	4	78 ± 10	12	6	67 ± 11	1	17	6 ± 5	7	11	39 ± 11	3	15	17 ± 9
MBX18	15	3	83 ± 9	10	8	56 ± 12	3	15	17 ± 9	12	6	67 ± 11	6	12	33 ± 11
MBX20	15	3	83 ± 9	11	9	55 ± 11	7	11	39 ± 11	16	2	89 ± 7	11	7	61 ± 11
MO1	1	7	14 ± 12	0	8	0	2	5	29 ± 17	8	0	100	8	0	100
ACR	8	0	100	8	0	100	8	0	100	0	8	0	0	8	0
WCS	0	8	0	0	8	0	0	8	0	0	8	0	0	8	0

[a] Treatments consisted of atrazine at 1000 g ai ha^{-1}, lactofen at 110 g ai ha^{-1}, imazamox at 44 g ai ha^{-1}, and glyphosate at 630 g ae ha^{-1} (0.75x) or at 1260 g ae ha^{-1} (1.5x)

[b] Values shown represent the percentage of resistant individuals ± the standard error.

Table 4.3 Linkage data for first run. Not all resistance data were available for each plant. Resistance frequencies were calculated for each herbicide in the absence of any other type of selection. Plants resistant to Herbicide 1 were then tested for resistance to Herbicide 2, and the observed resistance ratios were compared with those expected based on the resistance frequencies calculated for that same herbicide in the absence of any other type of selection. All pair-wise comparisons are shown. A single degree of freedom chi-square test was performed to test for significant deviations from expected ratios, and p-values were calculated. Deviations were considered significant for $p \leq 0.05$. Frequency of resistance to atrazine and glyphosate was determined through herbicide screening, while frequency of resistance to ALS and PPO inhibitors was determined solely by the use of molecular markers.

				Herbicide 2															
				Glyphosate						Atrazine						PPO			
	Resistance Freq.			Obs		Exp				Obs		Exp				Obs		Exp			
Herbicide 1	R	S	%R[a]	R	S	R	S	χ^2	p	R	S	R	S	χ^2	P	R	S	R	S	χ^2	p
ALS	105	53	66 ± 4	58	30	54	34	0.8	0.37	47	15	51	11	1.8	0.18	94	11	68	37	28.2	<0.001
PPO	102	56	65 ± 4	57	30	54	33	0.4	0.53	45	13	47	11	0.4	0.53						
Atrazine	80	18	82 ± 4	44	32	47	29	0.5	0.48												
Glyphosate	85	53	62 ± 4																		

[a] Values shown are percent resistant individuals ± the standard error.

Table 4.4 Linkage data for second run. Not all resistance data were available for each plant. Resistance frequencies were calculated for each herbicide in the absence of any other type of selection. Plants resistant to Herbicide 1 were then tested for resistance to Herbicide 2, and the observed resistance ratios were compared with those expected based on the resistance frequencies calculated for that same herbicide in the absence of any other type of selection. All pair-wise comparisons are shown. A single degree of freedom chi-square test was performed to test for significant deviations from expected ratios, and p-values were calculated. Deviations were considered significant for $p \leq 0.05$. Frequency of resistance to atrazine and glyphosate was determined through herbicide screening, while frequency of resistance to ALS and PPO inhibitors was determined solely by the use of molecular markers.

				Herbicide 2																	
				Glyphosate						Atrazine						PPO					
	Resistance Freq.			Obs		Exp				Obs		Exp				Obs		Exp			
Herbicide 1	R	S	%R[a]	R	S	R	S	χ^2	p	R	S	R	S	χ^2	P	R	S	R	S	χ^2	p
ALS	63	24	72 ± 5	22	33	21	34	0.08	0.78	33	8	34	7	0.17	0.68	60	3	44	19	19.3	<0.001
PPO	61	26	70 ± 5	21	33	20	34	0.08	0.78	31	7	31	7	0.00	1.00						
Atrazine	48	10	83 ± 5	19	29	18	30	0.09	0.76												
Glyphosate	30	50	38 ± 5																		

[a] Values shown are percent resistant individuals ± the standard error.

Table 4.5 Summary of linkage data, showing p-values for the first two runs of linkage tests, with the p-value for run one on top and the p-value for run two on the bottom. Resistances were considered linked for $p \leq 0.05$. Inset in lower right corner shows the numbers of plants resistant and susceptible to ALS inhibitors and PPO inhibitors. Due to similarity between runs, plants from both runs were pooled to obtain this count data. That linkage exists between ALS and PPO resistance can almost be inferred simply by inspection of these numbers.

	GLY	ATR	PPO
ALS	0.37	0.18	<.0001**
	0.78	0.68	<.0001**
PPO	0.53	0.53	
	0.78	1.00	
ATR	0.48		
	0.76		

	PPO-R	PPO-S
ALS-R	154	14
ALS-S	9	68

Table 4.6 Results of the final test for linkage between ALS and PPO resistance. Resistance frequencies in this run were determined by screening the plants with either imazamox (ALS) or lactofen (PPO). Molecular markers were not utilized in this run.

				Herbicide 2										
				ALS						PPO				
				Obs		Exp				Obs		Exp			
Herbicide 1	R	S	%R[a]	R	S	R	S	χ^2	p[b]	R	S	R	S	χ^2	p
ALS	69	22	76 ± 4							67	2	47	22	26.7	<<0.001
PPO	65	31	68 ± 5	64	1	49	16	18.7	<<0.001						

[a] Values shown are percent resistant individuals ± the standard error.

[b] <<0.001 indicates a p-value much smaller than 0.001.

CHAPTER 5

CONFIRMATION OF FOUR-WAY RESISTANCE IN AN ILLINOIS WATERHEMP POPULATION

5.1 Abstract

In 2006 and 2007, farmers from two different locations in Illinois reported a failure to control waterhemp with glyphosate. Seed heads were collected from surviving females from both fields in order to test these populations for glyphosate resistance. Seeds were pooled among females from an individual field, and separate glyphosate dose-response experiments were performed on plants from both populations. Results of these studies indicate high levels of glyphosate resistance are present in both fields. Subsequent field studies suggested that these populations may be resistant to multiple herbicides and, thus, greenhouse experiments were performed to investigate this possibility. Plants from both populations were screened with atrazine, acifluorfen, lactofen, and imazamox. One population was found to be resistant to acetolactate synthase (ALS) inhibitors in addition to glyphosate. The other glyphosate-resistant population was also found to be resistant to ALS inhibitors, protoporphyrinogen oxidase (PPO) inhibitors, and photosystem II inhibitors—specifically to triazines. This is the first waterhemp population to be reported with resistance to four herbicide modes of action. Based on these results, a question was raised as to whether this four-way resistance was only on the population level, or whether the population may contain individual plants that were resistant to all four herbicides. Thus, experiments were performed to identify four-way resistant individuals by sequentially screening plants with all four herbicides. Treatments consisted of either a mixture of imazamox and atrazine followed by (fb) a mixture of glyphosate and lactofen, or of a soil-

applied treatment of imazethapyr fb atrazine fb a glyphosate-lactofen mix. Control treatments were implemented to test the efficacy of each herbicide individually and in combination with other herbicides, and four-way resistant individuals were successfully identified through such screening. Finally, an investigation into the resistance mechanisms in these populations was undertaken. ALS resistance was found to be due to a target-site mutation consisting of the substitution of leucine for tryptophan at amino acid position 574 (W574L) in ALS in both populations. PPO resistance was found to be due to the previously reported deletion of a glycine codon at amino acid residue 210 (ΔG210) in *PPX2*. Triazine resistance was found not to be due to a target site mutation in *psbA*, and may perhaps be due to metabolism of the herbicide within the plants. The mechanism of glyphosate resistance also was investigated in the four-way resistant population. No evidence was found for gene amplification of *EPSPS* in resistant plants. Sequencing of *EPSPS* indicated a proline to serine substitution at position 106 (P106S) in the *EPSPS* enzyme, but this mutation did not cosegregate with resistance, indicating that some other mechanism conferring resistance to glyphosate must be present in this population. Results of this study should be cause for alarm for farmers, as the spread of this resistance could severely limit post emergence herbicide options for waterhemp control in soybean.

5.2 Introduction

Waterhemp is a small-seeded summer annual plant, which is indigenous to North America (Sauer 1955), and specifically to the midwestern United States (US). Originally found mainly along riverbanks and pond margins (Sauer 1957), in the past 20 years this species has advanced from relative obscurity to become ranked as one of the worst weeds in midwestern corn [*Zea Mays* L.] and soybean [*Glycine max* (L.) Merr.] fields (Hager et al. 2002).

Before this shift in prevalence, waterhemp already possessed several "weedy" characteristics which could potentially allow for it to become a problem in agronomic cropping systems. Waterhemp is a C_4 plant, and this photosynthetic system allows it to grow rapidly under high light and high temperature conditions, as well as to better tolerate drought when compared with C_3 species, such as soybean (Hopkins and Hüner 2004). Waterhemp is also a prolific seed producer, with plants capable of producing at least 1 million seeds under ideal growing conditions (Steckel et al 2003). Further, 1–3% of these seeds may remain viable in the soil seed bank for up to 17 years (Burnside et al. 1996). However, despite these characteristics, it was most likely a shift in production practices that ultimately led to this species becoming a major weed.

Several factors are thought to have contributed to this shift in prevalence. One such factor is the relatively recent shift toward reduced-tillage or no-tillage cropping systems. Such practices favor the growth of small seeded plants, as the seeds are allowed to remain at the soil surface where they can easily grow after germination (Buhler 1992; Hager et al. 1997). Another factor contributing to the recent increase in prevalence of waterhemp throughout the Midwest is related to an increased reliance on post emergence herbicides for weed control, which often have little if any residual activity in the soil (Hager et al. 1997). This, when combined with the fact that waterhemp seeds exhibit varying levels of dormancy, leading to a prolonged germination period and causing the seeds to germinate in flushes throughout much of the growing season, means that multiple post emergence herbicide applications are often necessary for season-long control of this species (Hager et al. 1997). Jasieniuk et al. (1996) reported that increased frequency of herbicide applications leads to increased selection pressure for herbicide-resistant weed biotypes,

and this appears to have been the case with waterhemp, as it has evolved resistance to several herbicides within the past two decades.

Waterhemp was first reported to have evolved resistance to triazine herbicides in 1990 (Anderson et al. 1996). Triazines are members of a larger class of herbicides that inhibit photosystem II (PS II) by competing with plastoquinone (Q_B) for its binding site on the D1 protein in the PS II pathway. This competition ultimately results in blockage of electron transport through the pathway, and leads to the production of singlet oxygen, which causes lipid peroxidation and the destruction of cell membranes (Hess 2000). These herbicides were used as early as 1956 (Patzoldt et al. 2003), and they remain popular due to their broad-spectrum weed control and soil-residual activity. Since their introduction, however, 68 species have evolved resistance to these herbicides worldwide (Heap 2010).

Waterhemp has been found to have at least two different mechanisms conferring resistance to triazines. One such mechanism is a single nucleotide substitution in the *psbA* gene which encodes for the D1 protein (Foes et al. 1998). This mutation results in the substitution of a glycine for a serine at amino acid position 264 (G264S), which results in a change in the Q_B binding site on the protein, reducing the herbicide's affinity for the site, thereby eliminating competition between Q_B and the herbicide and thus allowing the plant to survive herbicide treatment. This type of resistance, because it is due to a mutation in a gene located in the chloroplast, is maternally inherited.

Evidence of a second triazine resistance mechanism in waterhemp was reported by Patzoldt et al. (2003). Although the specific mechanism has not yet been elucidated, the authors reported that the resistance was not due to an altered target site, and that the resistance was nuclear inherited. Nuclear inherited triazine resistance has been reported in velvetleaf [*Abutilon*

theophrasti Medik.] and was found to be due to metabolism of the herbicide within the plant (Gronwald et al. 1989). Perhaps this same mechanism is responsible for conferring triazine resistance in waterhemp, but to date the true mechanism is unknown.

Resistance to acetolactate synthase (ALS) inhibiting herbicides was first reported in Illinois waterhemp in 1993 (Heap 2010). ALS inhibitors were first commercialized in 1982, and became quite popular due to their ability to control a broad spectrum of weeds, as well as their residual activity in the soil, low use rates, and low mammalian toxicities (Tranel and Wright 2002). When first marketed, these herbicides were effective tools for controlling waterhemp. However, ALS resistance in waterhemp is now so common that these herbicides are no longer recommended for waterhemp control in Illinois (Hager and Sprague 2003).

ALS inhibiting herbicides work by ultimately blocking the production of branched-chain amino acids, preventing susceptible plants from synthesizing proteins, thereby resulting in plant death. To date, six naturally occurring amino acid substitutions have been shown to confer resistance to ALS inhibitors (Tranel and Wright 2002; Whaley et al. 2007), and three of these have been discovered to confer target-site ALS resistance in waterhemp (Patzoldt et al. 2007). One of the most common substitutions, and one which confers a high level of cross-resistance to multiple families of ALS inhibiting herbicides is that of substitution of a leucine for tryptophan at amino acid position 574 (W574L) in ALS (Foes et al. 1998; Patzoldt et al. 2002) . This mechanism is nuclear inherited and thus can be spread through pollen as well as by seed movement.

In 1998, a waterhemp population in Illinois was found to be resistant to both triazines and ALS inhibiting herbicides, representing the first waterhemp population containing resistance to two herbicide families (Foes et al. 1998). This event was followed in 2002 by the discovery of an

Illinois waterhemp population that had evolved resistance to three different herbicide families—namely triazines, ALS inhibitors, and protoporphyrinogen oxidase (PPO) inhibiting herbicides (Patzoldt et al. 2005). This was the first case of PPO resistance in waterhemp in Illinois, as well as the first case of three-way resistant waterhemp ever reported (Heap 2010).

PPO inhibiting herbicides work by binding to protoporphyrinogen oxidase (Protox) and blocking the final step of the heme and chlorophyll biosynthesis pathway (Beale and Weinstein 1990; Duke et al. 1991). In the absence of such herbicides, this enzyme catalyzes the transformation of protoporphyrinogen IX (Protogen IX) to protoporphyrin IX (Proto IX). When the herbicide is present, however, and this reaction is blocked, Protogen IX begins to accumulate in the chloroplast and then leaks out into the cytoplasm. Once in the cytoplasm, Protogen IX is converted to Proto IX just as it would have been by Protox inside the chloroplast. However, in the cytoplasm, the newly formed Proto IX reacts with oxygen and light and, without the protective antioxidants found in the chloroplast, produces singlet oxygen, which causes lipid peroxidation and destroys cell membranes, thereby resulting in plant death.

Resistance to PPO inhibitors is conferred by a codon deletion in *PPX2*, which encodes for the Protox enzyme. This codon deletion results in the deletion of a glycine in Protox at position 210 (ΔG210) (Patzoldt et al. 2006), which is near the herbicide binding site of this enzyme. This mutation confers resistance to all three families of PPO inhibiting herbicides (Patzoldt et al. 2005), and is unique in the fact that this is the first codon deletion implicated in conferring resistance to herbicides. To date, this is the only known mechanism for resistance to PPO inhibitors in waterhemp (Lee et al. 2008).

With the evolution of ALS resistance in waterhemp, PPO inhibiting herbicides became important as they provided the only remaining option for effective post emergence chemical

control of waterhemp in soybean in the mid 1990s. Had this herbicide remained the only option, resistance to this herbicide in waterhemp would likely have become widespread relatively quickly. However, 1996 marked a revolution in post emergence chemical weed control in soybean with the commercialization of glyphosate-resistant soybeans.

Glyphosate is a broad-spectrum systemic herbicide that effectively controls many broadleaf and grass weeds—both annuals and perennials. This herbicide works by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is important in the biosynthesis of aromatic amino acids (Bradshaw et al. 1997). Thus, by inhibition of this enzyme, the herbicide prevents treated plants from synthesizing proteins, leading to plant death. Following the commercialization of glyphosate-resistant soybeans in 1996, the use of glyphosate increased dramatically from 2.5 million kg yr^{-1} before 1995 to 30 million kg yr^{-1} by 2002 (Young 2006). With increased use of glyphosate came increased selection pressure for glyphosate-resistant weeds, and the first glyphosate-resistant waterhemp biotype was reported in Missouri in 2004 (Legleiter and Bradley 2008).

Glyphosate resistance has been shown to be conferred through several mechanisms in other species, including reduced translocation of glyphosate from the treated leaves and an altered target site (Powles and Preston 2006). The most common target-site mutation found to confer glyphosate resistance consists of a substitution for proline at position 106 (P106) in EPSPS, and resistant plants from several species have been found to contain serine, threonine or alanine at this position (Wakelin and Preston 2006; Jasieniuk et al. 2008; Baerson et al. 2002). More recently, a Palmer amaranth [*Amaranthus palmeri* S. Wats.] population was found to be resistant to glyphosate due to gene amplification of *EPSPS* (Gaines et al. 2010). To date, the glyphosate resistance mechanism in waterhemp has yet to be determined.

In 2006 and 2007, farmers from different locations in Illinois reported failures to control waterhemp with glyphosate. Subsequent field studies suggested that these populations may contain multiple resistance (resistance to more than one herbicide). The purpose of this study was to determine whether these populations were resistant to glyphosate, as well as to investigate potential multiple resistance—specifically, resistance to triazines, ALS inhibitors and PPO inhibitors—by conducting studies in the greenhouse. If either of these populations were found to be resistant to all of these herbicides, this would mark the first occurrence of four-way resistance in a waterhemp population, and it could signify the onset of a very serious problem for post emergence herbicide control of waterhemp in soybean, especially if these populations contained individual plants that were resistant to all four of these herbicides.

5.3 Materials and Methods

5.3.1 Plant culture

All plants used in this study were grown from seeds sown in a 12 cm x 12 cm x 5 cm container in a medium consisting of a 3:1:1:1 mixture of commercial potting mix[1] to soil to peat to sand. When seedlings reached the two-leaf stage, they were transplanted into individual 6 cm x 4 cm x 5 cm inserts in 24 cm x 48 cm x 5 cm flats containing the previously mentioned growth medium. Unless otherwise indicated, when plants reached 5 cm in height they were transplanted to 12 cm square pots containing 700 ml of growth medium, where they were allowed to grow until completion of the experiment. Plants were fertilized as needed using a slow-release complete fertilizer[2], and the plants were grown in the greenhouse under mercury halide and sodium vapor lamps that provided a minimum photon flux of 800 µmol m^{-2} s^{-1} at the plant canopy in addition to the light incident from the sun. The lamps were programmed for a 16-h

photoperiod, and the greenhouse was maintained at temperatures of 22 C at night and 28 C during the day.

5.3.2 Herbicide application

All herbicide applications for this study were made using a compressed air, moving nozzle spray chamber with an adjustable platform and equipped with an 80015EVS even flat spray nozzle[3]. The nozzle was maintained at approximately 45 cm above the plant canopy. The sprayer was calibrated to deliver 187 L ha^{-1} of water at 207 kPa. Plants were returned to the greenhouse immediately after spraying.

5.3.3 Waterhemp populations

In 2006 a farmer in Fayette County, Illinois reported a failure to control waterhemp with glyphosate. In the fall of that year, seed heads were collected from 28 surviving female waterhemp plants. Samples were allowed to dry at room temperature, and seeds were then manually harvested and stored at 4 C until needed. For all greenhouse herbicide resistance confirmation experiments, seeds were pooled by collecting approximately equal numbers of seeds from all females and were stratified. The stratification procedure consisted of the seeds first being surface sterilized by a 10 min treatment with 1:1 bleach: water solution. Afterward, the seeds were washed twice with sterilized deionized water, suspended in 0.15% (w/w) agarose, and were then stored for at least two weeks at 4 C to break seed dormancy. This population is hereafter referred to as the FCG population (for Fayette County Glyphosate-Resistant).

In 2007 a farmer from Brown County in West-Central Illinois reported a failure to control waterhemp with glyphosate. Seed was collected from this population for testing in the

greenhouse as was done with the FCG population. Seed heads were collected from 10 female plants in the fall of 2007 and dried at room temperature. Before greenhouse testing, subsamples of equal numbers of seeds from each female were collected and pooled to form what will be referred to as the BCG population (for Brown County Glyphosate-Resistant). After pooling, seeds were stratified as described above.

5.3.4 Glyphosate dose-response

The FCG and BCG populations were screened for glyphosate-resistance in separate experiments. An Illinois waterhemp population designated as Adams County Resistant (ACR) which has been described previously (Patzoldt et al. 2005), was used as the susceptible control, and a waterhemp population from Missouri, designated as MO1, which has also been described previously (Legleiter and Bradley 2008), was used as the resistant control in both experiments. The experiments were performed as a completely randomized design (CRD) with at least four replicates, and both experiments were repeated.

In both experiments, plants were grown in the greenhouse as previously described until they reached 5 cm in height, at which time plants used in the FCG dose response experiment were transplanted to the previously described 12 cm square 700 ml pots, while plants used for the BCG dose response experiments were transplanted to 10 cm round 400 ml pots containing the previously described growth medium. In both experiments, when plants reached 10–15 cm in height they were treated with glyphosate[4]. In the first experiment the populations treated were ACR, MO1 and FCG, while in the second experiment the treated populations were ACR, MO1 and BCG. In each of the experiments, glyphosate was applied at rates of 0, 52.5, 105, 210, 420,

840, 1680, 3360, and 6720 g ae ha^{-1}, and each treatment included 2.5% (v/v) ammonium sulfate (AMS)[5] as well as 0.25% (v/v) non-ionic surfactant (NIS)[6].

Plants were rated visually for injury level at 16 days after treatment (DAT) on a scale of 0 to 10, with a 0 indicating no herbicide injury and a 10 indicating a dead plant. After visual ratings had been recorded, the above-ground biomass was harvested, dried for at least 72 hours in a cabinet dryer at 60 C, and dry weights recorded for each plant. In addition to the treatments previously mentioned, at least four plants of each biotype were harvested at the time of herbicide treatment, dried for at least 72 hours and weighed for comparison with the treated plants. This allowed for the determination of the amount of biomass accumulated after treatment. The responses of the FCG and BCG populations were compared with those of the ACR and MO1 populations in order to evaluate the level of glyphosate resistance.

5.3.5 Dose-response statistical analysis

The data used in the glyphosate dose-response analysis were the percent of untreated control accumulated dry matter between 0 and 16 DAT. To calculate this quantity, first the mean dry weight of plants harvested at the time of treatment (or 0 DAT) was calculated for each biotype, and this mean weight was then subtracted from the measured dry weights of each treated plant of the corresponding biotype that was harvested at 16 DAT. The accumulated dry matter of all treated plants then was converted to a percent of untreated control dry matter accumulation by calculating the mean dry matter accumulation for untreated plants of each biotype harvested at 16 DAT and dividing the accumulated dry weight of each plant by the mean dry matter accumulation of the untreated control of the corresponding biotype, as in

$$P_{ijk} = \frac{\left(F_{ijk} - \bar{I}_i\right)}{\bar{C}_i} \times 100 \qquad [1]$$

where P_{ijk} is the percent of control accumulated dry matter of the jth replicate of the ith biotype receiving the kth dose, F_{ijk} is the measured dry weight at 16 DAT of the jth replicate of the ith biotype receiving the kth dose (the *final* dry weight), $\bar{I}_i$ is the mean dry weight of plants of the ith biotype harvested at 0 DAT (the *initial* dry weight), and $\overline{C_i}$ is the mean accumulated dry weight of untreated control plants of the ith biotype harvested at 16 DAT.

In three of the four dose-response runs, a four-parameter log-logistic equation

$$y = f(x) = C + \frac{D - C}{1 + \exp[b(\log(x) - \log(GR_{50}))]} \quad [2]$$

was used as in Seefeldt et al. (1995) to calculate the herbicide dose at which after-treatment dry matter accumulation was reduced to 50% of that the untreated control plants at 16 DAT (GR_{50}) for each biotype. Here *y* represents the percent of control accumulated dry matter for each plant, *x* represents the herbicide dose, *D* represents the mean response of the untreated control, *C* represents the mean response at high doses, and *b* represents the slope of the curve around the GR_{50}. In the second run of the FCG glyphosate dose response experiment, a four-parameter Weibull model was used to fit the data as in

$$y = f(x) = C + \frac{D - C}{\exp\{\exp[b(\log(x) - GR_{50})]\}}. \quad [3]$$

Here the parameters are identical to those described above for the four-parameter log-logistic equation. The Weibull model is an asymmetric curve used to model dose-response data for which the initial decent is rapid with a more gradual approach toward the lower limit (Knezevic et al. 2007), and this model was used to fit the data and to calculate GR_{50} values in the second run of the FCG glyphosate dose response because it provided a better fit than did the log-logistic curve.

The R statistical software program with the drc extension package (Knezevic et al. 2007) was used to fit curves to the data to calculate GR_{50} values, and the data were tested for interactions between experimental runs. If run interactions were significant, the data were not pooled across runs.

5.3.6 Multiple herbicide resistance screen

Due to reports of failure to control the FCG and BCG waterhemp populations with other herbicides in subsequent field tests, greenhouse tests were conducted to investigate other types of herbicide resistance in both populations. Plants were grown in the greenhouse as previously described until they became 10–15 cm tall, at which time they were screened for resistance to triazines, PPO-inhibitors, and ALS-inhibitors by separate treatments with atrazine[7], lactofen[8], acifluorfen[9], and imazamox[10]. All atrazine, lactofen and acifluorfen spray solutions included 1% (v/v) crop oil concentrate (COC)[11], and all imazamox solutions contained 1% (v/v) COC and 2.5% (v/v) AMS. Each experiment was performed as a CRD and included at least four replicates for each treatment and experiments were repeated at least once.

In both runs of the FCG multiple resistance screen, plants were treated with atrazine at 200 and 1000 g ai ha^{-1}, acifluorfen at 30 and 90 g ai ha^{-1}, lactofen at 110 g ai ha^{-1}, and imazamox at 44 g ae ha^{-1}. ACR was used as the resistant control population, and a population designated as Wayne County Susceptible (WCS), which was previously described by Patzoldt et al. (2005), was used as the susceptible control. Plants from the BCG population were included in the second run.

In subsequent runs, only the BCG population was screened for multiple resistance. Atrazine was applied only at the 1000 g ai ha^{-1} rate, and acifluorfen was no longer applied. ACR

and WCS were again used as control populations, but MO1 was also included in these subsequent runs, and treatments did not always include all three herbicides (atrazine, lactofen and imazamox) in a single run. The third multiple resistance screen consisted of treatments with atrazine and lactofen. Run four consisted of only a treatment with atrazine. Run five consisted of separate treatments of atrazine, lactofen, and imazamox at the previously indicated rates, as well as a treatment of glyphosate at 3360 g ae ha^{-1} in order to investigate the glyphosate resistance mechanism discussed later. In all runs of the multiple resistance experiments, the response of the FCG and/or BCG plants for each treatment was compared with that of the controls at 16 DAT, and plants were scored as either R or S.

5.3.7 Multiple resistance statistical analysis

Percent survival was calculated by counting the number of surviving plants from a particular herbicide treatment and dividing by the total number of plants of the corresponding biotype that received that treatment. Percent survival for each biotype and run was analyzed using PROC GLM in SAS[12], and data from different runs were pooled if there was no significant run effect. Standard errors for the categorical survival data were calculated according to

$$\sigma_{\hat{\pi}} = \sqrt{\frac{\hat{\pi}(1-\hat{\pi})}{n}} \qquad [4]$$

as shown by Ott and Longnecker (2001), where $\sigma_{\hat{\pi}}$ is the standard error of the mean survival percentage, n is the number of plants of a particular biotype that all received the same herbicide treatment, and $\hat{\pi}$ is the observed survival percentage (an estimate of the true value) calculated as

$$\hat{\pi} = \frac{a}{n} \qquad [5]$$

where *a* is the number of plants of a particular biotype that survived treatment with a particular herbicide.

Percent of control dry weights were calculated similarly to the percent of control accumulated dry matter calculation described in the glyphosate dose-response statistical analysis section above. Specifically, percent of control dry weights were calculated as

$$P_{ijk} = \frac{W_{ijk}}{\overline{C}_i} \quad [6]$$

where P_{ijk} is the percent of untreated control dry weight of the jth replicate of the ith biotype receiving the kth herbicide treatment, W_{ijk} is the measured dry weight at 16 DAT of the jth replicate of the ith biotype receiving the kth herbicide treatment, and $\overline{C}_i$ is the mean dry weight of untreated control plants of the ith biotype harvested at 16 DAT. Mean percent of control dry weights were calculated as simple averages of the P_{ijk}s for each biotype and treatment.

5.3.8 Screen for four-way resistant individuals from the BCG population

Based on results of the multiple resistance screen, an attempt was made to identify individuals from the BCG population that were resistant to four herbicide modes of action. The strategy was to screen each plant with four different herbicides while attempting to avoid potential antagonism among the herbicides, in which the effect of one herbicide may reduce or inhibit the herbicidal effect of another herbicide either directly or indirectly (Green 1989). Thus, the BCG plants screened in this experiment (at least 90 in each run) were screened with sequential herbicide applications. In the first run of the experiment, individuals from the BCG population were first screened for ALS resistance and triazine resistance by application of a mix of imazamox at 44 g ae ha^{-1} and atrazine at 1000 g ai ha^{-1}. This mixture was applied when

seedlings were 5 cm tall and it included 1% (v/v) COC and 2.5% (v/v) AMS. Atrazine and imazamox were also applied separately and in combination to the control biotypes ACR and WCS to test the efficacy of each herbicide. Plants were rated as resistant or susceptible at 9 DAT, and resistant plants were then subjected to treatment with a mix of glyphosate and lactofen at 4200 g ae ha^{-1} and 110 g ai ha^{-1}, respectively. The herbicide solution also contained 2.5% (v/v) AMS, as well as 0.25% (v/v) NIS. Glyphosate and lactofen were also applied separately and in combination to the control biotypes WCS, ACR, and MO1 to test the efficacy of each herbicide. Plants were rated as R or S at 14 days after the final mix treatment.

In the second run of the experiment, BCG plants were screened for ALS-resistance using a soil-applied treatment of imazethapyr[13] at 1400 g ai ha^{-1}, corresponding to 20 times the normal field use rate. This high rate was chosen based on the results of preliminary work, which showed that rates of 210 g ai ha^{-1} (3 times the field rate) failed to control WCS, the susceptible biotype, presumably due to high levels of organic matter in the soil. Surviving seedlings were transplanted to 12 cm square 700 ml pots when they were 5 cm tall, were allowed two days to recover from transplanting, and were then subjected to treatment with atrazine at 1000 g ai ha^{-1}, which included 1% (v/v) COC. BCG seedlings were compared with atrazine-treated WCS and ACR seedlings in order to identify triazine-resistant plants. BCG plants surviving treatment with atrazine were then allowed to grow until they were 10–15 cm tall, at which time they were treated with a mix of glyphosate[14] and lactofen at 4200 g ae ha^{-1} and 110 g ai ha^{-1}, respectively. The herbicide solution also contained 2.5% (v/v) AMS, as well as 0.25% (v/v) NIS. Again, glyphosate and lactofen were also applied separately and in combination to control biotypes WCS, ACR, and MO1 to test the efficacy of each herbicide. Plants were rated as R or S at 14 days after the final herbicide treatment.

5.3.9 Molecular investigation of resistance mechanisms

5.3.9.1 Sample preparation

A 100 mg sample of meristematic leaf tissue was collected from each BCG plant used in the study as well as several ACR and WCS plants when they reached approximately 5 cm in height. The samples were placed in 96-well plates, each of which was kept on ice until it had been filled, at which time the plate was then covered and wrapped with parafilm, and then placed in storage at -80 C until needed. Additional 100 mg tissue samples were also collected from FCG plants that survived treatment with imazamox, as well as from the few FCG plants surviving treatment with acifluorfen and atrazine in the multiple resistance screens. These samples were placed in 1.5 ml microcentrifuge tubes and were also stored at -80 C until needed.

Total DNA was extracted from meristematic leaf tissue by using a modified hexadecyltrimethyl-ammonium bromide (CTAB) protocol from Doyle and Doyle (1990). Extracted DNA was resuspended in 50 µl of TE buffer. DNA was then quantified using a spectrophotometer[15] and was then diluted to 50–100 ng μL^{-1} using sterile deionized water. The DNA was then stored at -20 C until needed.

5.3.9.2 Triazine resistance

DNA was extracted from samples collected from one FCG plant which survived treatment with atrazine, from three atrazine-resistant BCG plants and three atrazine-susceptible BCG plants, as well as from three WCS and three ACR plants. The plants were investigated for target-site resistance. A fragment of *psbA* (the gene encoding the D1 protein) was amplified via polymerase chain reaction (PCR) using primers described in Foes et al. (1998). PCRs consisted of 50–100 ng DNA, 0.2 mM of each dNTP[16], 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and

reverse primers[17], and one unit of Taq polymerase[18] with a 1x concentration of supplied buffer in a final volume of 20 µL. The samples were subjected to an initial denaturation step of 94 C for 2 min followed by 30 cycles of 30 s of denaturation at 94 C, 30 s of annealing at 50 C, and 30 s of extension at 72 C. Results of the PCR were then checked via gel electrophoresis by running 5 µL of each product in a 1.2% (w/v) agarose gel. Desired products were identified as bands on the gel corresponding to fragments 277 base pairs (bp) in length. The remaining 15 µL of PCR product in samples with successful amplification of *psbA* were then purified using a PCR purification kit[19], and the products were sequenced as previously described (Foes et al. 1998) using the forward primer. Sequence data were aligned and compared with sequences of known triazine-resistant and triazine-susceptible *Amaranthus hybridus* plants from Genbank (accession K01200) using sequence analysis software[20]. *Amaranthus hybridus* was used because the sequence of *psbA* is well-conserved across species, and this species is the closest relative to waterhemp for which sequence data for the target-site mutation in *psbA* conferring resistance to atrazine is available from Genbank.

5.3.9.3 ALS inhibitor resistance

The field populations were investigated for the presence of target-site resistance to ALS-inhibitors via the common tryptophan-to-leucine substitution at amino acid position 574 (W574L mutation). Genomic DNA was extracted as indicated above from four ALS-resistant FCG plants, three ALS-resistant BCG plants and three ALS-susceptible BCG plants. Region B of *ALS* was amplified via PCR using the same primers as were used by Foes et al. (1998). PCRs contained 50–100 ng DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and reverse primers, and one unit of Taq polymerase with a 1x concentration of supplied buffer in a

final volume of 20 µL. The samples were subjected to an initial denaturation step of 95 C for 3 min followed by 35 cycles of denaturation at 95 C for 30 s, annealing at 56 C for 1 min, and extension at 72 C for 1 min 30 s. The samples were then subjected to a final extension step of 72 C for 5 min. Results of the PCR were then checked via gel electrophoresis by running 5 µL of each product in a 1.2% (w/v) agarose gel. Desired products were identified as bands on the gel corresponding to fragments 451 bp in length. The remaining 15 µL of PCR product in samples with successful amplification of *ALS* were then purified using a PCR purification kit as discussed above, and the products were sequenced as previously described using the reverse primer. Sequence data were aligned and compared with the *ALS* sequences of WCS and ACR that have been submitted to Genbank (accessions EF157818 and EF157819, respectively) using sequence analysis software. WCS is susceptible to ALS-inhibiting herbicides, while ACR is resistant, and the resistance mechanism in ACR is the W574L mutation.

5.3.9.4 PPO inhibitor resistance

The field populations were investigated for the presence of the glycine codon deletion in *PPX2L* at position 210 (ΔG210), which to this point is the only known mutation to confer resistance to PPO-inhibiting herbicides in waterhemp (Patzoldt et al. 2006; Lee et al. 2008). Genomic DNA was extracted from three FCG plants surviving treatment with acifluorfen as well as from three PPO-resistant BCG plants surviving treatment with lactofen and three PPO-susceptible BCG plants using the same method discussed above. A fragment of *PPX2L* was amplified using PCR with forward primer 5'-GAG AAA ACA CAA TGC TAC TGA A-3' and reverse primer 5'-ACA GCC TCC AGA AAA TGT TG-3'. PCRs consisted of 50–100 ng DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and reverse primers, and

one unit of Taq polymerase with a 1x concentration of supplied buffer in a final volume of 20 µL. The samples were subjected to an initial denaturation step of 95 C for 2 min followed by 39 cycles of denaturation at 95 C for 30 s, annealing at 55 C for 30 s, and extension at 72 C for 45 s. PCR products were checked via gel electrophoresis by running 5 µL of each product in a 1.2% (w/v) agarose gel. Desired products were identified as bands on the gel corresponding to fragments 785 bp in length. The remaining 15 µL of PCR product in samples with successful amplification of *PPX2L* were then purified using a PCR purification kit as discussed above, and the products were sequenced as previously described using both the forward and reverse primers in separate sequencing reactions. Sequences were then analyzed using sequence analysis software and were then aligned and compared with those of WCS and ACR that have been submitted to GenBank (accessions DQ394875 and DQ394876, respectively).

5.3.9.5 Glyphosate resistance

5.3.9.5.1 Test for gene amplification of *EPSPS*

To date, the mechanism of glyphosate resistance in waterhemp has not been elucidated. However, it has been shown that the mechanism of glyphosate resistance in a related species, Palmer amaranth, is due to elevated copy number of the gene encoding *EPSPS* (Gaines et al. 2010), which is the target site for glyphosate. The authors hypothesized that the same mechanism may confer resistance to glyphosate in waterhemp, and so *EPSPS* copy number was investigated in this species.

Genomic DNA was extracted from 20 BCG plants screened in the final multiple resistance screen discussed above with varying phenotypes from glyphosate-resistant to glyphosate-susceptible again using the modified CTAB procedure. After re-suspending the DNA

in TE buffer, the DNA was quantified and then diluted to 10–50 ng μL^{-1} using sterile deionized water. Relative copy number of *EPSPS* in resistant plants was compared with that of susceptible plants using quantitative real-time PCR (qPCR) as described in Chapter 3.

5.3.9.5.2 Sequencing *EPSPS*

As another investigation into the mechanism of glyphosate resistance in the BCG population, a fragment of *EPSPS* containing amino acid position 106 was sequenced. This region is of interest as a proline to serine substitution at position 106 (P106S) in *EPSPS* has been shown to confer at least moderate levels of glyphosate resistance in several other species such as goosegrass [*Eleusine indica* (L.) Gaertn.] (Baerson et al. 2002), Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot] (Jasieniuk et al. 2008), and rigid ryegrass [*L. rigidum* Gaudin] (Wakelin and Preston 2006).

DNA extracted for use in qPCR from three resistant and three susceptible BCG plants was used for sequencing *EPSPS* as was DNA from 3 WCS plants and 2 greenhouse-confirmed glyphosate-resistant MO1 plants. *EPSPS* was amplified using primers that were originally designed for use in qPCR: EPSF1 (5'-ATG TTG GAC GCT CTC AGA ACT CTT GG-3') and EPSR8 (5'-TGA ATT TCC TCC AGC AAC GGC AA-3') (Gaines et al. 2010). PCRs consisted of 50–100 ng DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and reverse primers, and one unit of Taq polymerase with a 1x concentration of supplied buffer in a final volume of 20 µL. The samples were subjected to touchdown PCR, consisting of an initial denaturation step of 95 C for 2 min followed by 10 cycles of denaturation at 95 C for 1 min, annealing for 30 s, and extension at 72 C for 45 s. The annealing temperature was 65 C in the first cycle and was reduced by 1 C in each of the next cycles until it reached 55 C, after which

the samples were subjected to 24 more cycles of denaturation at 95 C for 1 min, annealing at 55 C for 30 sec, and extension at 72 C for 45 sec. A final extension step of 72 C for 4 min was included and samples were then held at 4 C. PCR products were checked via gel electrophoresis by running 5 µL of each product in a 1.0% (w/v) agarose gel. Desired products were identified as bands on the gel corresponding to fragments of 195 bp. The remaining 15 µL of PCR product in samples with successful amplification of *PPX2L* were then purified using a PCR purification kit as discussed above, and the products were sequenced as previously described using both the forward and reverse primers in separate sequencing reactions. *EPSPS* sequences were then analyzed using sequence analysis software and were aligned and compared with those of WCS and ACR that have been submitted to GenBank (accessions FJ869881 and FJ869880, respectively).

5.4 Results and Discussion

5.4.1 Glyphosate resistance

5.4.1.1 Resistance in the FCG population

Due to the presence of an interaction between runs, the data for the FCG glyphosate dose response runs were not pooled. The FCG population demonstrated a high level of glyphosate resistance in the first run of the glyphosate dose response experiment, with a GR_{50} value of 1400 g ae ha^{-1} (Table 5.1). When compared with the GR_{50} of ACR of 55 g ae ha^{-1}, this implies that the FCG population demonstrated a 25-fold level of resistance (calculated by dividing the GR_{50} of FCG by that of ACR) , which was even higher than that of MO1—a population previously confirmed to be resistant to glyphosate (Legleiter and Bradley 2008). In the second run, the estimated GR_{50} value for the FCG population was much lower, at 190 g ae ha^{-1}, while the

corresponding estimated GR_{50} values for ACR and MO1 were 110 g ae ha^{-1} and 1700 g ae ha^{-1}, respectively. This corresponds to a decrease in the calculated resistance ratio of the FCG population to just 1.7-fold resistance in the second run, while the resistance ratio for MO1 remained nearly constant, at 12-fold and 15-fold in runs 1 and 2, respectively. However, these values may be misleading.

Figure 5.1 shows the calculated percent of control accumulated dry matter versus glyphosate dose for ACR, FCG and MO1. In run 1 (Panel A), there is a wide separation between the response of FCG and that of ACR, particularly at 105, 210, and 840 g ae ha^{-1}. However, in run 2 (Panel B), as seen from the wide error bars on the means of the FCG response to glyphosate at 210 and 840 g ae ha^{-1}, differences between the FCG and ACR responses to glyphosate were nearly undetectable at these doses, which may have caused a low estimate for the GR_{50} value of the FCG population in this run, as the error bars at both doses span the 50% of control mark. Interestingly, the error bars for MO1 and ACR populations are noticeably smaller in both runs. As error bars serve as an indication of variability among experimental replicates, in dose-response experiments large error bars may be indicative of a population segregating for response to herbicide, and that is precisely what can be seen in the FCG population.

Figure 5.2 shows pictures of the responses of all plants of each of the three biotypes (ACR, FCG, and MO1 from left to right) treated with glyphosate in both runs of the dose-response experiment. Immediately obvious from inspection of these pictures is a difference in the response of the ACR population between runs at low doses. In run one, some injury is apparent even at the 52.5 g ae ha^{-1} dose, while in run two, ACR injury was only easily apparent beginning at the 210 g ae ha^{-1} dose. This probably contributed to the GR_{50} value of ACR in the second run being twice that of ACR in the first run (Table 5.1). Also obvious in the pictures in

Figure 5.2 is segregation in the response of the FCG population. In run one, 1 FCG plant was killed at the 52.5 g ae ha^{-1} dose. Segregation is visible in run two at 210 g ae ha^{-1}, which was the lowest dose that killed ACR plants, indicating that the FCG plants which were killed at that dose were susceptible to glyphosate. It is interesting to note that in run two at 210 g ae ha^{-1} and at 840 g ae ha^{-1}, 3 of the FCG plants look similar to ACR, while the other 3 plants look similar to MO1, which demonstrated a generally uniform response to glyphosate except at the higher doses in run one. This range of responses in the FCG population led to the wide error bars seen in Figure 5.1 Panel B, which nearly span the difference between the responses of MO1 and that of ACR at both of those doses.

However, despite the obvious segregation in the FCG population (which was not quantified) in both runs as well as the decreased response of ACR to glyphosate at low doses in run two, ACR was well controlled at high doses in both runs of the experiment, while some FCG plants survived at these doses and displayed similar responses to MO1 plants. This clearly demonstrates glyphosate resistance in the FCG population, and it can be seen easily in Figure 5.3, which displays representative responses of resistant plants from the FCG population compared with the average response of plants from the ACR and MO1 populations for both runs. Again the difference in response of ACR in run 1 (Panel C) and run 2 (Panel F) is apparent, with ACR being more difficult to control in run 2. The response of MO1 was similar between runs, with plants appearing only slightly more injured at the 6720 g ae ha^{-1} dose (corresponding to 8 times the field use rate) in the first run (Panel A) than in the second (Panel D). The representative responses of non-susceptible FCG plants (Panels B and E) was similar to the responses of MO1 plants in both runs, indicating a high level of glyphosate resistance in a significant number of individuals in the FCG population. With continued selection pressure from glyphosate

applications in the field from which this population was obtained, the frequency of glyphosate-resistant individuals can be expected to increase (Jasieniuk et al. 1996).

5.4.1.2 Resistance in the BCG population

In the BCG glyphosate dose response experiment, data from each run were again analyzed separately due to the presence of run interactions. In run one of this experiment, the estimated GR_{50} value of the BCG population was 1400 g ae ha^{-1}, compared with 1200 and 95 g ae ha^{-1} for MO1 and ACR, respectively, indicating that the BCG population is 15-fold more resistant than the ACR population (Table 5.2). Similar to the results of the FCG glyphosate dose response, the resistance ratio of the BCG population was much lower in the second run, at 3.1-fold more resistant than ACR. The same trend was shown by the MO1 population, with a resistance ratio of 13-fold in the first run and 3.3-fold more resistant than ACR in the second. However, investigation of the GR_{50} values of the biotypes in both runs indicate that this difference in resistance ratios is attributable largely to a higher GR_{50} value of ACR in run two, with which resistance ratios of BCG and MO1 were calculated, as the GR_{50} values of BCG and MO1 were similar between runs.

Investigation of Figure 5.4, which shows the mean percent of control accumulated dry matter versus glyphosate dose for ACR, BCG, and MO1 in both runs, allows for visualization of the reason for the higher estimated GR_{50} of ACR in run two versus that of run one. In the second run of the experiment (Panel B), the response of ACR was similar to that of BCG and MO1 until doses of 420 g ae ha^{-1} and higher, while in the first run (Panel A) a difference in response between ACR and that of BCG and MO1 is apparent at doses as low as 52.5 g ae ha^{-1}, indicating that ACR was more easily controlled in the first run.

This fact is also visible in Figure 5.5, in which ACR demonstrates a high level of injury at doses of 420 g ae ha^{-1} and higher in the first run of the experiment, while in the second run of the experiment ACR was not consistently controlled until doses of 1680 g ae ha^{-1} and higher. Another interesting aspect of this experiment that is visible in Figure 5.5 is the near-uniform response of the BCG population to glyphosate, with one plant at most out of six showing a high level of injury at doses of 3360 g ae ha^{-1} and lower in both runs of the experiment. In the first run, only two BCG plants show a response similar to that of ACR at the 6720 g ae ha^{-1} dose, with the remaining four plants behaving similar to MO1 at that dose. This is in contrast to the higher level of segregation for glyphosate resistance in the FCG population that was apparent in Figure 5.2.

Similar to the FCG population, however, the BCG population displayed a clear resistance to glyphosate. This is obvious in Figure 5.5 in the pictures taken of plants in runs one and two at higher doses, and at 3360 and 6720 g ae ha^{-1} in particular. Those doses provided fairly consistent control of ACR plants in both runs, while the majority of the BCG plants subjected to those doses survived and showed phenotypes similar to those of MO1. Figure 5.6 shows representative responses of resistant BCG plants to different glyphosate doses in both runs of the experiment compared with representative responses of ACR and MO1 at the same doses in both runs. The difference in the response of ACR to glyphosate between runs is clear (Panels C and F), with ACR surviving higher doses in the second run than in the first. The MO1 plants show a similar response across runs (Panels A and D) with plants perhaps just slightly taller at all but the highest two doses in run two when compared with run one. The BCG population demonstrated a similar response both within and between runs (Panels B and E). Taken collectively, these data indicate that the BCG population is nearly uniformly resistant to glyphosate.

5.4.1.3 General observations on inter- and intra-run variability in response to glyphosate

The response pictures of the waterhemp populations used in the above dose response experiments, and that of ACR in particular, showed clear variation both across runs of a particular experiment, as well as between whole experiments. Several factors may be important in contributing to such variation. These factors are addressed in Appendix A, but they will be briefly discussed here.

One of the most important factors in causing differences in waterhemp's response to herbicide is pot size (data not shown). Specifically, plants grown in smaller pots tend to be less responsive to herbicides than do plants grown in larger pots, and this may be part of the reason that ACR required somewhat higher doses of glyphosate to be killed in the BCG dose response (plants grown in 400 ml pots) than in the FCG dose response (plants grown in 700 ml pots). This is presumably due to plants in smaller pots becoming stressed because of the limited space for root growth by the time the plants reach the usual spraying height of 10–15 cm. Other factors observed in the past to cause differences in response to herbicide, which probably were not involved in causing the differences noted in the previous experiments, are fertilizer and watering frequency. Namely, plants with sufficient fertilizer and that are watered at least two times per day tend to be under less stress than unfertilized or under-watered plants, and these plants tend to be more sensitive to herbicide than stressed plants (data not shown). However, there apparently are some other important environmental factors which have not yet been identified in affecting waterhemp's response to herbicide, and such factors must have caused the differences observed between runs of both the FCG and the BCG glyphosate dose response studies. For instance, one potential factor in causing such variation between runs may be the amount of natural sunlight present in the days after herbicide treatment. Although supplemental lighting was present in the

greenhouse as mentioned in the Materials and Methods section, on cloudy days there is obviously less light in the greenhouse room than on sunny days. It would be interesting to keep a record of light conditions and investigate whether or not some correlation exists between the amount of light and the response of waterhemp plants to herbicide. Presumably, other factors may also be important, such as relative humidity, stress during transplanting, or greenhouse pest control. However, the determination of the importance of such factors in affecting waterhemp's herbicide response is beyond the scope of this study.

5.4.2 Multiple resistance

In all multiple resistance screens, the susceptible control, WCS, was inconsistently controlled by acifluorfen, particularly at the 30 g ai ha^{-1} rate, but also at the 90 g ai ha^{-1} rate, as can be seen in Figure 5.7 (Panels A and B), in which one WCS plant even survived treatment at the 90 g ai ha^{-1} rate. This picture shows the best control of WCS achieved by acifluorfen out of two applications at two different rates, and therefore the response to this herbicide was excluded from analysis in the multiple resistance screens. However, because the FCG plants did appear less injured than WCS by treatment with acifluorfen, tissue samples were collected from 3 FCG plants surviving these treatments, in order to test for the known PPO resistance-conferring mutation in *PPX2*, the results of which are discussed later. Atrazine applications at 200 g ai ha^{-1} were also problematic, as WCS was never well controlled by such treatments, one instance of which is shown in Panel C of Figure 5.7. Thus, the 200 g ai ha^{-1} treatment of atrazine was also excluded from subsequent evaluations of multiple resistance in both biotypes.

5.4.2.1 Multiple resistance in the FCG population

The FCG population demonstrated nearly-uniform ALS resistance, with little or no resistance to triazines and PPO-inhibitors (Table 5.3). In fact, 82% of FCG plants (or 14 out of 17) survived treatment with imazamox, while only 5.9% and 12% of FCG plants survived treatment with atrazine and lactofen, respectively. This corresponded to 1 out of 17 plants surviving atrazine treatment and 2 out of 17 plants surviving lactofen treatment. It is worth noting that the FCG plants surviving treatment with lactofen did just that—they survived, but barely. The visual ratings for the two FCG plants surviving treatment with lactofen were 8 and 9 (data not shown), where a 10 indicates a dead plant, and a 0 indicates no injury. ACR plants subjected to the same treatment received injury ratings of between 1 and 3, with an average rating of 1.3, and thus were much healthier than the FCG plants, for which the mean visual rating was 9.8. The one FCG plant that survived treatment with atrazine, however, appeared very healthy and was given a visual rating of 1. This plant's phenotype was comparable with that of ACR plants receiving the same treatment, which all received visual ratings of 0 or 1, giving a mean rating of 0.5, while the mean rating for FCG plants was 9.2. This indicates a low frequency of triazine resistant individuals in the population, and little if any PPO resistance.

Figure 5.8 shows pictures of FCG plants treated with several herbicides in the first run of the experiment. In Panel A, the near-uniform resistance of this population to ALS inhibitors is apparent, with only one out of six FCG plants showing any obvious signs of injury due to treatment with imazamox. Panel B shows plants treated with atrazine, all of which were controlled in this run, except for ACR. The plants shown in Panel C were treated with lactofen, and again it can be seen that this population is largely susceptible to PPO inhibitors, as all six

FCG plants treated in the first run of the experiment were controlled with lactofen, while the resistant control plants (ACR) survived.

In addition to the tissue samples collected from the FCG plants that survived treatment with acifluorfen (which failed to completely kill WCS) described above, tissue samples were also collected from FCG plants surviving treatment with imazamox as well as from the one plant surviving treatment with atrazine in order to investigate the ALS and triazine resistance mechanisms in this population, the results of which are discussed later.

5.4.2.2 Multiple resistance in the BCG population

Unlike the results of the FCG multiple herbicide resistance screen, the BCG population showed fairly high levels of resistance to all three herbicides tested (Table 5.4). In the first run of the experiment, 58% of the BCG population was found to be resistant to atrazine, with 83% and 92% of the population resistant to lactofen and imazamox, respectively. The BCG plants that were resistant to atrazine were highly resistant, as can be seen in the visual rating data. Although nearly one-half of the BCG plants were killed by atrazine in this run, corresponding to visual ratings of 10, the mean visual rating for all 12 plants was only 4.9, indicating that the surviving plants displayed low levels of atrazine injury. The plants surviving treatment with lactofen were also highly resistant, with a mean visual rating of 3.2, compared with 1.3 for ACR and 10 for WCS. As for ALS resistance, nearly all of the BCG plants survived in the first run, and the mean visual rating was 2.8 for these plants, compared with 0.8 and 9.9 for ACR and WCS, respectively.

In subsequent screens, these numbers were found to be different in some cases—specifically the frequencies of PPO and ALS resistance, which were 38% and 55%, respectively

in later runs of the experiment. The proportion of triazine-resistant individuals remained constant, with a frequency of 61% found in subsequent screens. The control populations behaved largely as expected in all runs (WCS was controlled by all herbicides, while ACR survived treatment with each of the herbicides), with the exception of one ACR plant which was killed by treatment with lactofen, indicating slightly higher activity of this herbicide in later runs of the experiment. This can also be seen in the dry weight data, as the dry weight of lactofen-treated ACR plants in a later run was reduced to 7.1% of that of the untreated control plants. This could partially explain the lower percent survival figure for BCG plants treated with lactofen in subsequent runs. However, the controls for imazamox worked perfectly in all runs, with ACR surviving all applications, and all WCS being controlled by this herbicide, so the difference in BCG percent survival figures for imazamox should not be attributable to higher imazamox efficacy in later runs. In fact, investigation of the dry weight data indicates that ACR was less injured in subsequent screens, with the mean dry weights of treated plants being 99% of those of the untreated plants, compared with 89% in the first run of the experiment. Thus, it is surprising that fewer BCG plants survived imazamox in subsequent runs of the experiment. One potential explanation for the observed phenomenon is that this shift in ALS resistance frequencies was due to the use of a different BCG seed pool between the first multiple herbicide resistance screen (which was also used for both glyphosate dose-response runs) and the subsequent screens, as seeds from some of the females collected from the field were no longer available at the time of creation of the second BCG seed pool. This may also partly explain the decrease in frequency of PPO resistant individuals in the BCG population as well. However, despite this difference in resistance frequencies between the first screen and subsequent screens, the fact is that the BCG

population was found to contain significant frequencies of triazine, PPO, and ALS resistance in all runs of the multiple herbicide resistance experiment.

Figure 5.9 shows pictures taken of the final screen in this experiment. Panel A shows a high level of resistance to atrazine, with resistant BCG plants looking similar to the resistant control, ACR. In Panel B, BCG plants surviving imazamox treatment are shown, which again display a response similar to that of ACR. Panel C shows the results of a treatment with glyphosate at 3360 g ae ha^{-1}, or 4 times the field use rate. In this treatment, all WCS and ACR were controlled, and the response of the BCG plants is similar to that of plants from the glyphosate-resistant MO1 population. PPO resistance is documented Panel D, with BCG plants actually displaying fewer symptoms of lactofen injury than even those of the resistant control, which again was ACR in this case. Based on the resistance frequencies and the pictures showing comparison of BCG plants with resistant and susceptible control populations, the conclusion must be that this population contains resistance to four herbicides, each with a different site of and mode of action. This marks the first waterhemp population documented to contain such four-way resistance (Heap 2010). However, these results raise the question—does the BCG population contain *individual plants* that are resistant to each of the four herbicides?

5.4.3 BCG screen for four-way resistant individuals

The results of the four-way resistance screen on individual plants answer the previous question in the affirmative. In the first run of this experiment, 137 BCG plants at the 5 cm growth stage were treated with a mix of atrazine at 1000 g ai ha^{-1} and imazamox at 44 g ai ha^{-1} as described in the Materials and Methods. WCS and ACR plants at the same height were treated with both herbicides separately and in combination. WCS was controlled by all treatments, while

ACR survived each of the three treatments. 63 of the 137 BCG plants survived this treatment, and the survivors were then subjected to treatment with a mix of glyphosate at 4200 g ae ha^{-1} and lactofen at 110 g ai ha^{-1}. The high rate of glyphosate was chosen in order to eliminate uncertainties due to somewhat inconsistent control of ACR by glyphosate in the previously described dose-response experiments. Controls for this portion of the experiment consisted of WCS (which should be controlled by both glyphosate and lactofen), ACR (which should be controlled by glyphosate) and MO1 (which should be controlled by lactofen). Plants from each of these populations received treatments of each of the herbicides separately and in combination at the same rates as those used in the mix. ACR plants which had been subjected to the atrazine-imazamox mix were also treated with the glyphosate-lactofen mix to test for decreased response due to plant stress which may have been imposed by the initial herbicide treatment.

The controls worked as expected, with WCS and ACR controlled by glyphosate, WCS and MO1 controlled with lactofen, and all three biotypes controlled by the glyphosate-lactofen mix. Of the 62 BCG plants treated with the mix (1 of the 63 that survived the initial atrazine-imazamox mix was discarded due to non-uniformity), 41 survived, and thus were classified as four-way resistant individuals. These plants were then transferred to an empty greenhouse room and allowed to intermate in an attempt to create a uniform line of four-way resistant plants.

In the second run of the experiment, a soil-applied treatment of imazethapyr was used in place of the imazamox that had been used in the mix with atrazine in the first run. This was an important method of screening for ALS resistant individuals, due to the lack of control populations displaying resistance to ALS inhibiting herbicides or to atrazine, but not to both. More specifically, in the first run of the experiment the controls used during the application of the atrazine-imazamox mix were not effective in ruling out antagonism between these two

herbicides, as ACR is resistant to both, while WCS is susceptible to both. Therefore, one can imagine a scenario in which perhaps the presence of imazamox in a plant cell somehow antagonizes or inhibits the herbicidal effect of atrazine. If this were to happen, this effect would have been undetectable with the control populations used, as WCS could have died from imazamox alone. Thus, the fact that WCS was controlled by the atrazine-imazamox mix while ACR survived such treatment does not necessarily indicate that both herbicides were equally effective in the mix.

Thus, with the soil-applied treatment of imazethapyr in the second run of this experiment, this problem was avoided altogether. The imazethapyr treatment arrested the growth of recently-germinated WCS seeds, eventually leading to the death of the seedlings, while ACR seedling growth appeared uninhibited from such treatment. Therefore, BCG seedlings surviving this herbicide treatment were considered to be ALS resistant. When these seedlings reached 5 cm in height, they were treated with atrazine as described earlier, as were WCS and ACR seedlings of the same size. The controls responded to this treatment as expected, with WCS being controlled and ACR surviving. Of the 90 BCG seedlings in this run of the experiment treated with atrazine, 59 survived and were classified as both triazine- and ALS-resistant. When these surviving BCG plants reached 10–15 cm in height, they were treated with a glyphosate-lactofen mix as was used in the first run. Controls consisted of the same treatments and populations used in the first run of this experiment. The responses of all plants treated with the glyphosate-lactofen mix were evaluated at 14 DAT. The control treatments again worked as expected, and 17 of the 59 BCG plants treated with this glyphosate-lactofen mix survived and thus were classified as four-way resistant individuals. Three of these four-way resistant BCG individuals are shown in the far right row in Figure 5.10.

5.4.4 Resistance mechanisms

In both the FCG and the BCG populations, a single nucleotide substitution in *ALS* resulting in a W574L substitution in the ALS enzyme was discovered in resistant plants (Table 5.5). This mutation has been shown to confer broad cross-resistance to all families of ALS-inhibitors in several species (Tranel and Wright 2002; Patzoldt et al. 2002), and is believed to be the most common ALS resistance-conferring mutation present in waterhemp in Illinois (Patzoldt et al. 2002). Although other ALS resistance-conferring mutations may be present in these populations, the W574L mutation alone is sufficient for resistance, and the resistance mechanism was not investigated further.

BCG plants demonstrating resistance to lactofen, as well as an FCG plant that survived treatment with acifluorfen, were found to have *PPX2* alleles containing the ΔG210 mutation. This is the only mechanism known to confer resistance to PPO inhibiting herbicides in waterhemp to date (Patzoldt et al. 2006), and it is likely that this mutation is the cause of PPO resistance in plants from both of these populations. However, due to the apparent low levels of PPO resistance in the FCG population, alleles containing the ΔG210 mutation are expected to be rare in this population.

As for triazine resistance, no mutations were discovered in the *psbA* gene which encodes for the D1 protein involved in photosystem II. A mutation in amino acid residue 264 in *psbA* resulting in the substitution of a glycine for a serine (G264S) in the D1 protein has been shown to confer resistance to triazines in several species, including waterhemp (Foes et al. 1998). However, this is not the only mechanism conferring resistance to triazines in waterhemp. Although no other mechanisms have been elucidated in this species, evidence of at least one other mechanism that is not due to a target site mutation does exist (Patzoldt et al. 2003), which

may be the same mechanism conferring resistance to triazines in the BCG population, and to a few individuals in the FCG population.

The mechanism of resistance to glyphosate in both of these populations is unclear. Individuals from the FCG population have demonstrated limited gene amplification of *EPSPS* (up to a 4-fold increase in copy number over that of WCS) (Liu, Riggins, Tranel unpublished), which appears to be weakly correlated with glyphosate resistance. However, no *EPSPS* amplification whatsoever was observed in the BCG population after investigation of 20 individual plants displaying varying phenotypes from resistant to susceptible in response to glyphosate treatment at 3360 g ae ha^{-1}, which indicates that gene amplification is not the resistance mechanism in this population.

The results of sequencing *EPSPS* in the BCG population were rather interesting, as a single nucleotide polymorphism was found, which results in the substitution of a serine for a proline at position 106 (P106S) in the EPSPS enzyme. This mutation has been shown to confer moderate levels of glyphosate resistance in other species (Baerson et al. 2002; Wakelin and Preston 2006; Jasieniuk et al. 2008). However, even more interesting was the fact that this mutation was found not only in the three glyphosate-resistant plants for which *EPSPS* was sequenced, but it was also discovered in the three susceptible plants for which *EPSPS* was sequenced. This appears to indicate that this mutation does not confer resistance to glyphosate in the BCG population. However, perhaps the presence of some other factor along with this target-site mutation is required for plants to be resistant to glyphosate, but beyond the tests performed, identifying the mechanism of glyphosate resistance in the BCG population is beyond the scope of this study.

Although this mutation found in *EPSPS* did not lead to the discovery of the mechanism of glyphosate resistance in the BCG population, this finding may be of some use to weed scientists working with this population in the future. This polymorphism could potentially serve as a genetic marker, which could be valuable in inheritance studies of glyphosate-resistance as well as in experiments involving hybridization between waterhemp and other *Amaranthus* spp., among other uses. Although other markers do exist in waterhemp, this one may be valuable assuming that the polymorphism is a rare one. This is in contrast to the use of ALS resistance as a marker, which will likely become of more limited use in both field and greenhouse studies with the prevalence of ALS resistance—which can be transferred through pollen flow—in Illinois.

The data presented here demonstrate that glyphosate resistance has evolved in two waterhemp populations in Illinois. This fact alone is alarming due to the almost universal reliance on glyphosate for weed control in corn and soybean in the Midwest. However, the finding of resistance to other herbicides in these populations means even more limited options for chemical control of waterhemp—particularly in soybean. With the discovery of four-way resistant individuals in the BCG population, effective post emergence control options in this crop are now limited solely to glufosinate, which requires the use of glufosinate-resistant soybean. And based on the recent history of the evolution of herbicide resistance in waterhemp, it should be expected that by relying heavily on a new herbicide to control four-way resistant waterhemp, this species will soon evolve resistance to that one as well.

5.5 Future Work

As a final step in this study, it would be interesting to sequence *ALS* and *PPX2* from individual plants shown to be four-way resistant by screening with herbicide in order to confirm

that these plants contain both resistance mechanisms and to further rule out antagonism among herbicides. Investigation into the mechanism of glyphosate resistance in this population may provide some interesting information. It would also be interesting to cross glyphosate-resistant plants from the BCG population with MO1 plants and to test the level of glyphosate resistance in the progeny of such a cross. Additionally, crossing a four-way resistant BCG individual with plants from a susceptible population such as WCS and creating an F_2 population from such a cross could be instructive for investigations into linkage between the four types of herbicide resistance in the BCG population. For instance, in an extreme case of linkage among all four resistance types, one would expect to see all F_2 plants either resistant to all four herbicides or susceptible to all four herbicides, although such tight linkage is probably not the reality. Performing such crosses between BCG and WCS (or ACR) could also be instructive in investigation of the inheritance of glyphosate resistance in this population.

5.6 Sources of Materials

[1] LC1 professional growing mix, Sun Gro Horticulture Canada Ltd., 52130 RR 65, P.O. Box 189, Seba Beach, AB 70E 2BO Canada. Distributed by Sun Gro Horticulture Distribution Inc. 15831 N.E. 8th St., Suite 100, Bellevue, WA USA 98008.

[2] Scotts Osmocote Classic 13-13-13 slow-release fertilizer. The Scotts Company LLC, 14111 Scottslawn Rd., Marysville, OH 43041.

[3] TeeJet 80015EVS spray nozzle. TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187.

[4] Touchdown HiTech® Herbicide. Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419.

[5] N-PaK® AMS Liquid, Winfield Solutions, LLC, P.O. Box 64589, St. Paul, MN 55164-0589.

[6] Activator 90 Nonionic Surfactant. Loveland Products, Inc. PO Box 1286, Greeley, CO 80632.

[7] AAtrex® Nine-O. Syngenta International AG. Schwarzwaldallee 215 P.O. Box CH-4002, Basel, Switzerland.

[8] Cobra® herbicide. Valent U.S.A. Corporation. 1600 Riviera Ave., Suite 200, Walnut Creek, CA 94596-8025.

[9] Blazer® Postemergence Herbicide for Soybeans. United Phosphorus, Inc. 423 Riverview Plaza, Trenton, NJ 08611.

[10] Raptor® Herbicide. BASF Corporation. 26 Davis Drive, Research Triangle Park, NC 27709.

[11] Herbimax® Petroleum Oil-Surfactant Adjuvant. Loveland Products, Inc., P.O. Box 1286, Greeley, CO 80632.

[12] Statistical Analysis Software (SAS). SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC 27513.

[13] Pursuit® Herbicide. BASF Corporation. 26 Davis Drive, Research Triangle Park, NC 27709.

[14] MON 76255 40.2% ae Technical Grade Glyphosate. Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167.

[15] Nanodrop 1000 Spectrophotometer v3.7.1. Thermo Fisher Scientific Inc., 81 Wyman St., Waltham, MA 02454.

[16] Invitrogen 100 mM dNTP Set, PCR Grade, Invitrogen Corporation, 5791 Van Allen Way, P.O. Box 6482, Carlsbad, CA 92008.

[17] IDT Custom Oligos. Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241.

[18] GoTaq Flexi DNA Polymerase. Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711.

[19] QIAquick PCR Purification Kit. QUIAGEN, Inc. USA., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[20] Sequencher 4.9 Software. Gene Codes, 775 Technology Drive, Suite 100A, Ann Arbor, MI 48108.

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5.9 Tables and Figures

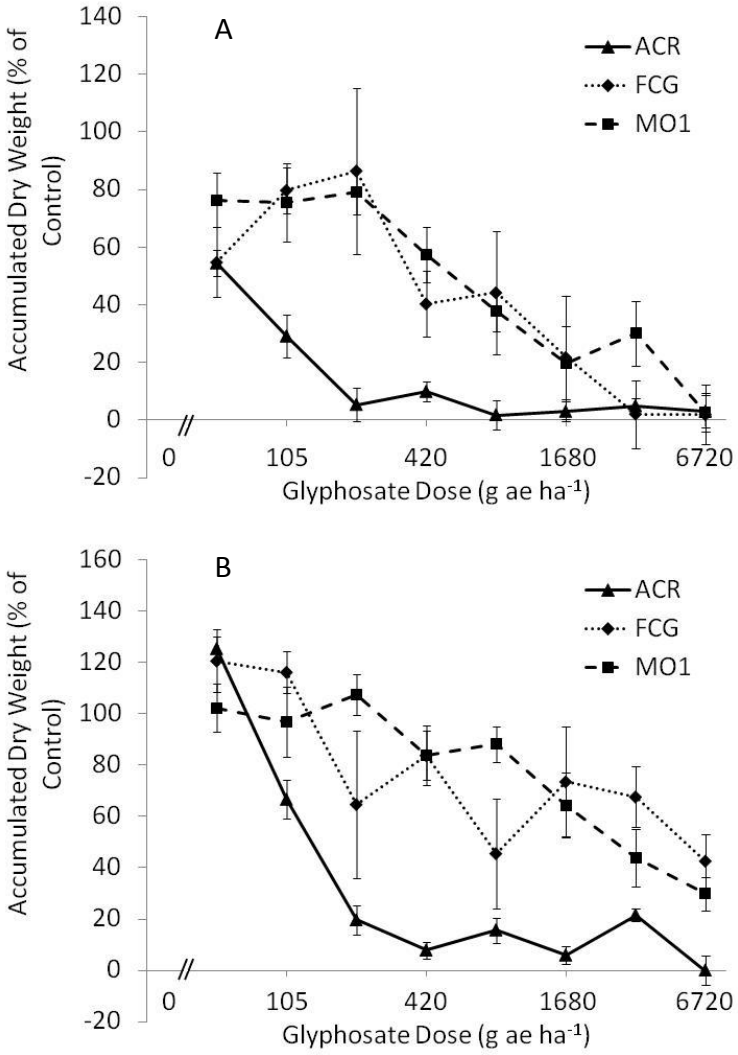

Figure 5.1 FCG glyphosate dose response results. Data presented are the mean percent of control dry matter accumulated in 16 DAT for each biotype at glyphosate doses of 52.5 g ae ha^{-1} and higher. Due to an interaction between runs, both runs 1 (A) and 2 (B) are shown separately. Error bars represent ± the standard error of the mean (SEM).

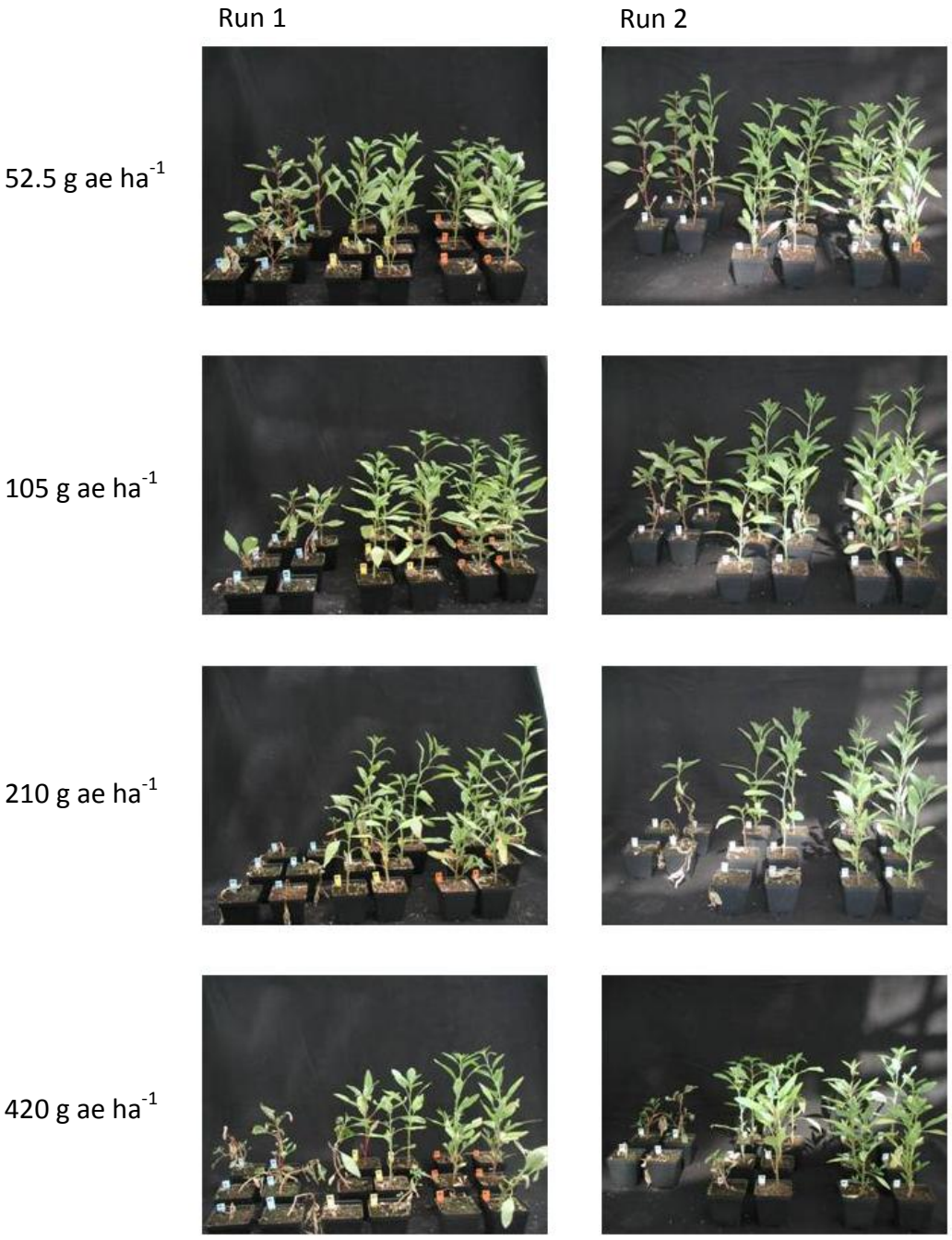

Figure 5.2 Pictures of FCG plants in rounds 1 and 2 of the glyphosate dose response experiment treated with glyphosate at 52.5, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha^{-1}. In all pictures biotypes are ACR, FCG and MO1 from left to right with two rows for each type, and plants have been sorted from most injured (front) to least injured (back).

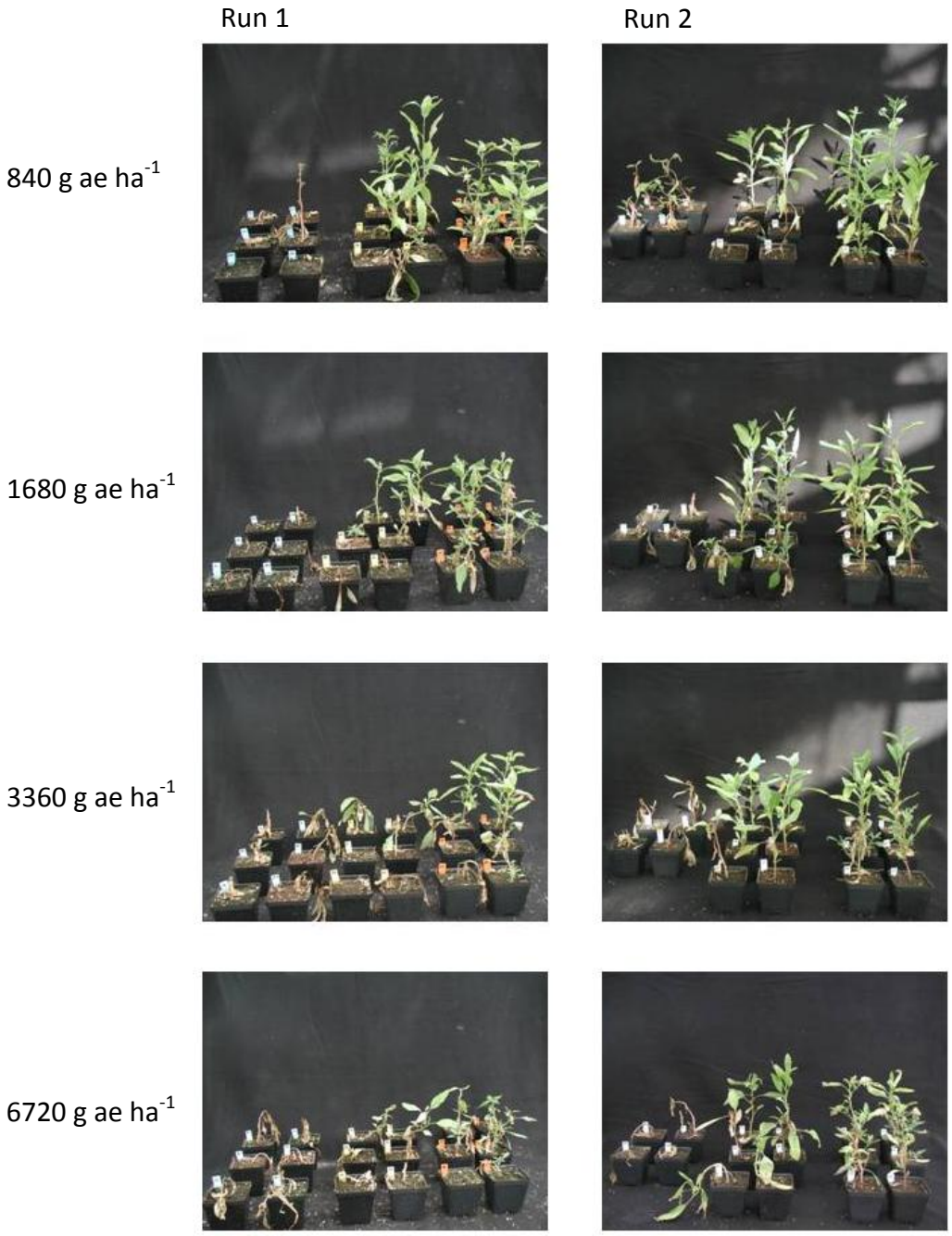

Figure 5.2 (cont.) Pictures of FCG plants in rounds 1 and 2 of the glyphosate dose response experiment treated with glyphosate at 52.5, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha^{-1}. In all pictures biotypes are ACR, FCG and MO1 from left to right with two rows for each type, and plants have been sorted from most injured (front) to least injured (back).

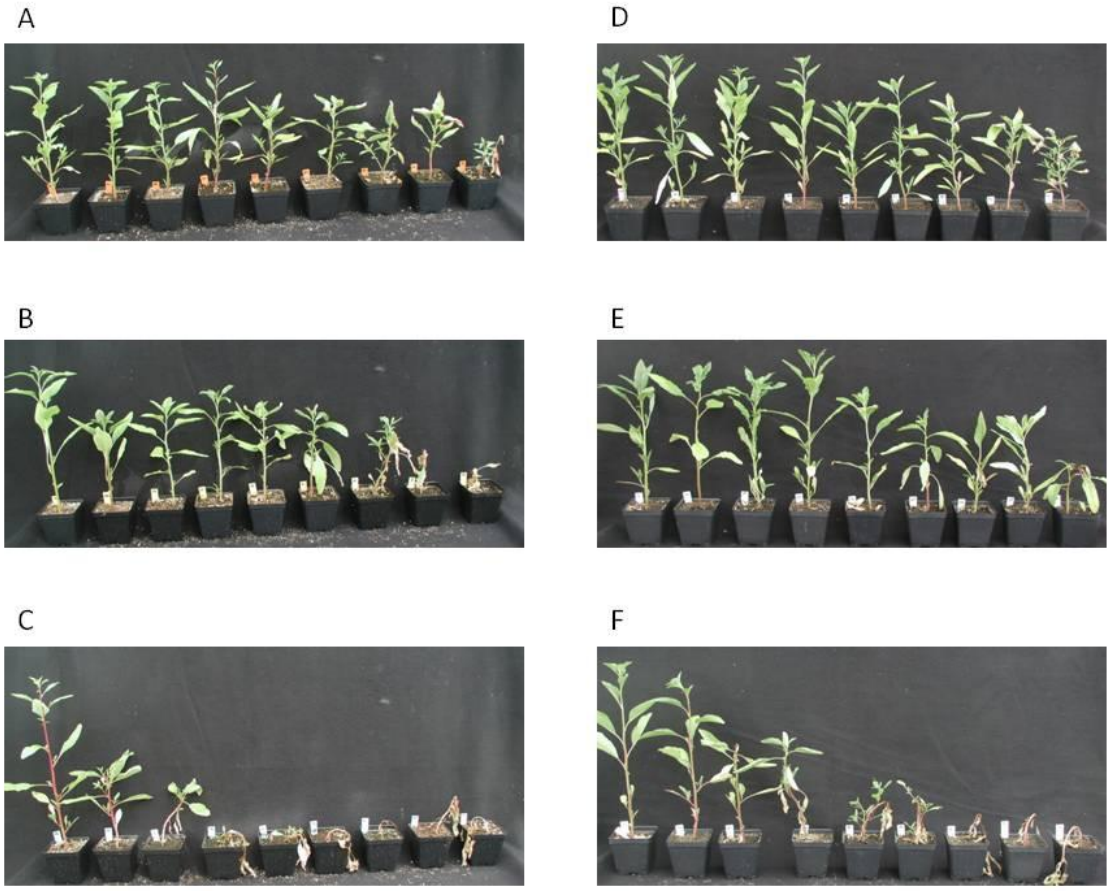

Figure 5.3 Pictures of response of resistant FCG plants at all doses of glyphosate. Doses displayed are 0, 52.5, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha^{-1} from left to right. Pictures A–C represent the response of MO1, FCG, and ACR, respectively, in the first run, while pictures D–F depict the response of MO1, FCG, and ACR, respectively, in the second run.

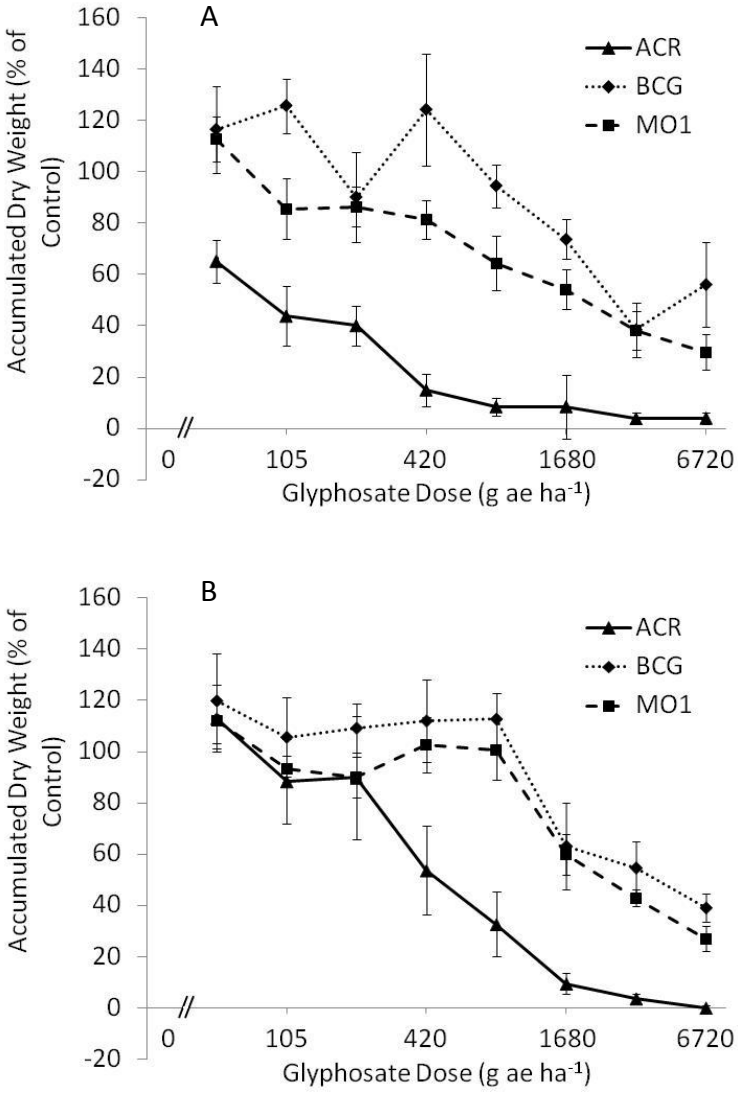

Figure 5.4 Results of the BCG glyphosate dose response. Data presented are the mean percent of control dry matter accumulated in 16 DAT for each biotype at glyphosate doses of 52.5 g ae ha^{-1} and higher. Due to an interaction between runs, both runs 1 (A) and 2 (B) are shown separately. Error bars represent ± SEM.

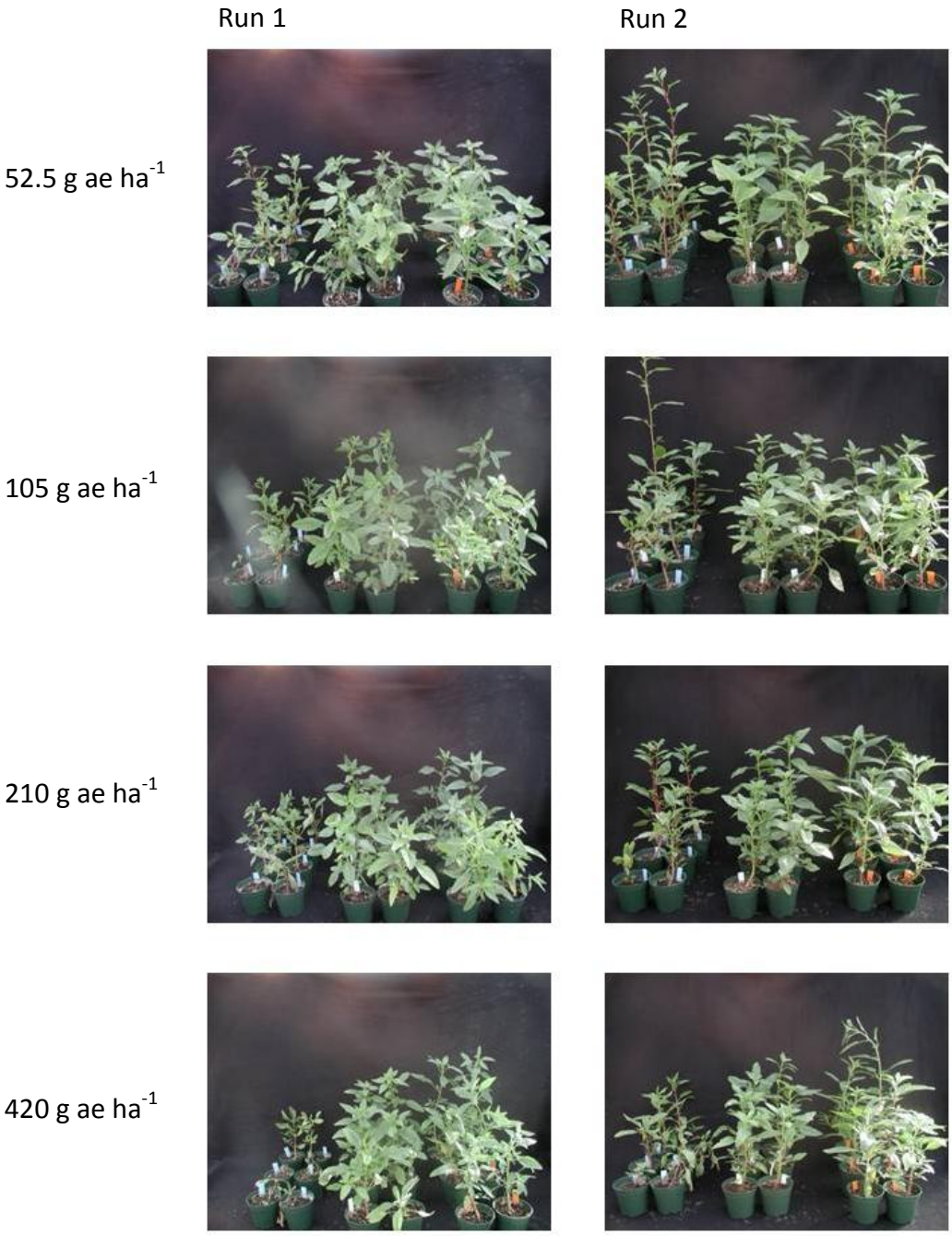

Figure 5.5 Pictures of BCG plants in rounds 1 and 2 of the glyphosate dose response experiment treated with glyphosate at 52.5, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha^{-1}. In all pictures biotypes are ACR, BCG and MO1 from left to right with two rows for each type, and plants have been sorted from most injured (front) to least injured (back).

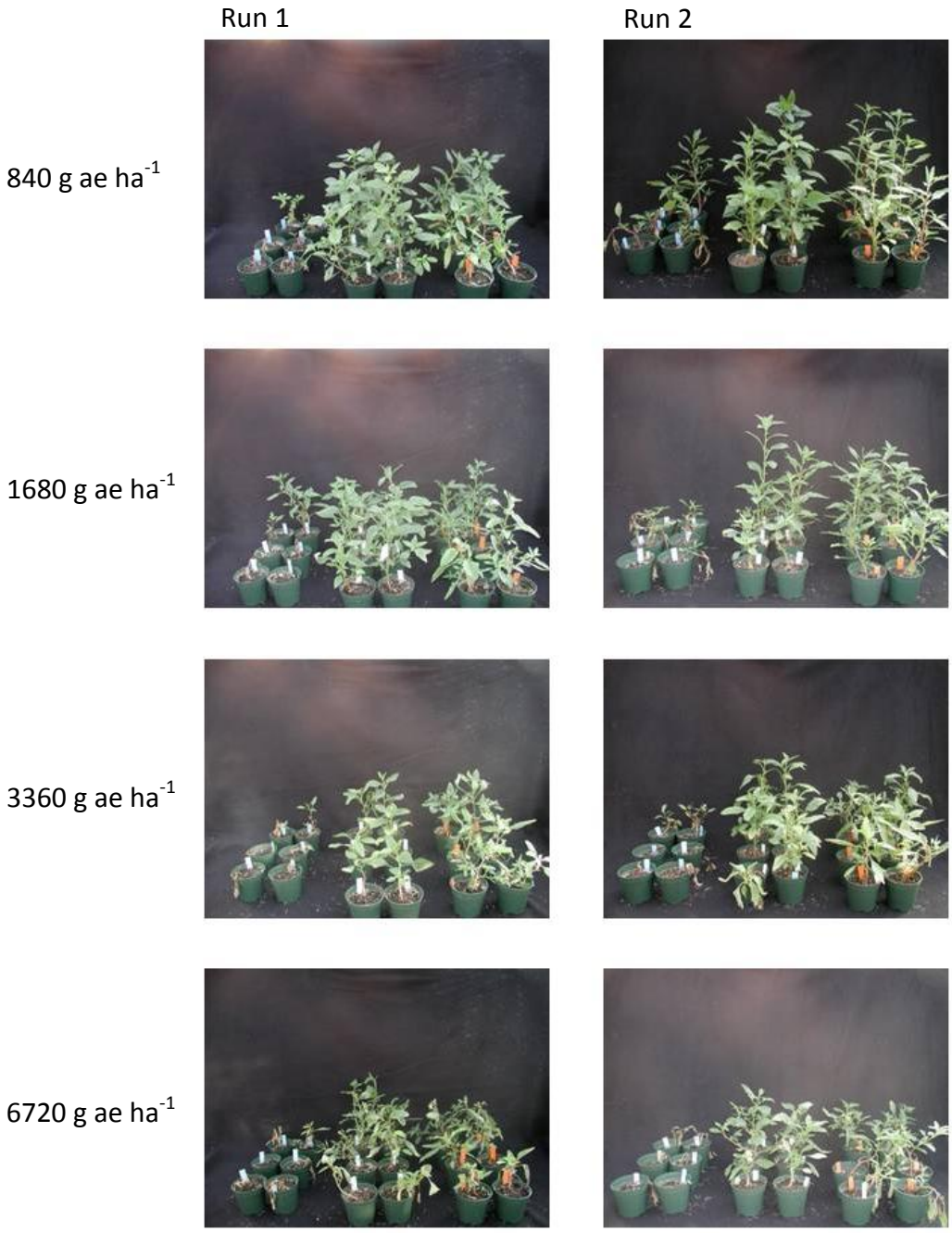

Figure 5.5 (cont.) Pictures of BCG plants in rounds 1 and 2 of the glyphosate dose response experiment treated with glyphosate at 52.5, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha^{-1}. In all pictures biotypes are ACR, BCG and MO1 from left to right with two rows for each type, and plants have been sorted from most injured (front) to least injured (back).

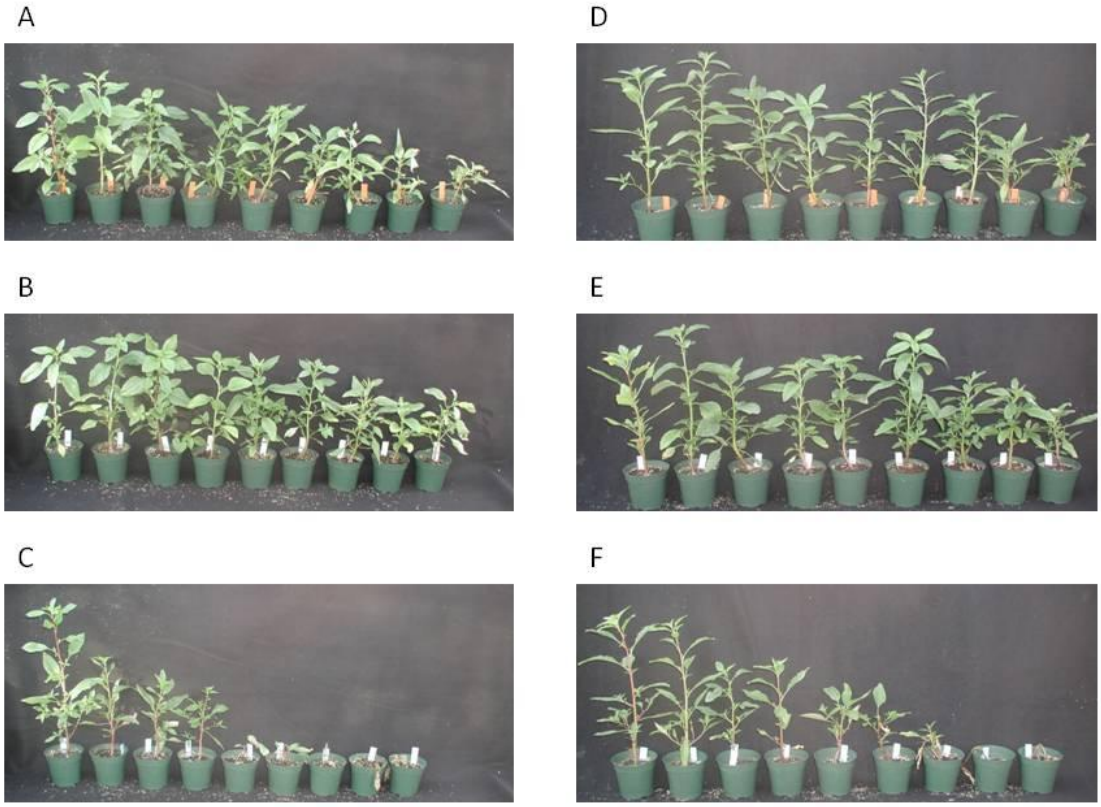

Figure 5.6 Pictures of response of resistant BCG plants at all doses of glyphosate. Doses displayed are 0, 52.5, 105, 210, 420, 840, 1680, 3360, and 6720 g ae ha^{-1} from left to right. Pictures A–C represent the response of MO1, BCG, and ACR, respectively, in the first run, while pictures D–F depict the response of MO1, BCG, and ACR, respectively, in the second run.

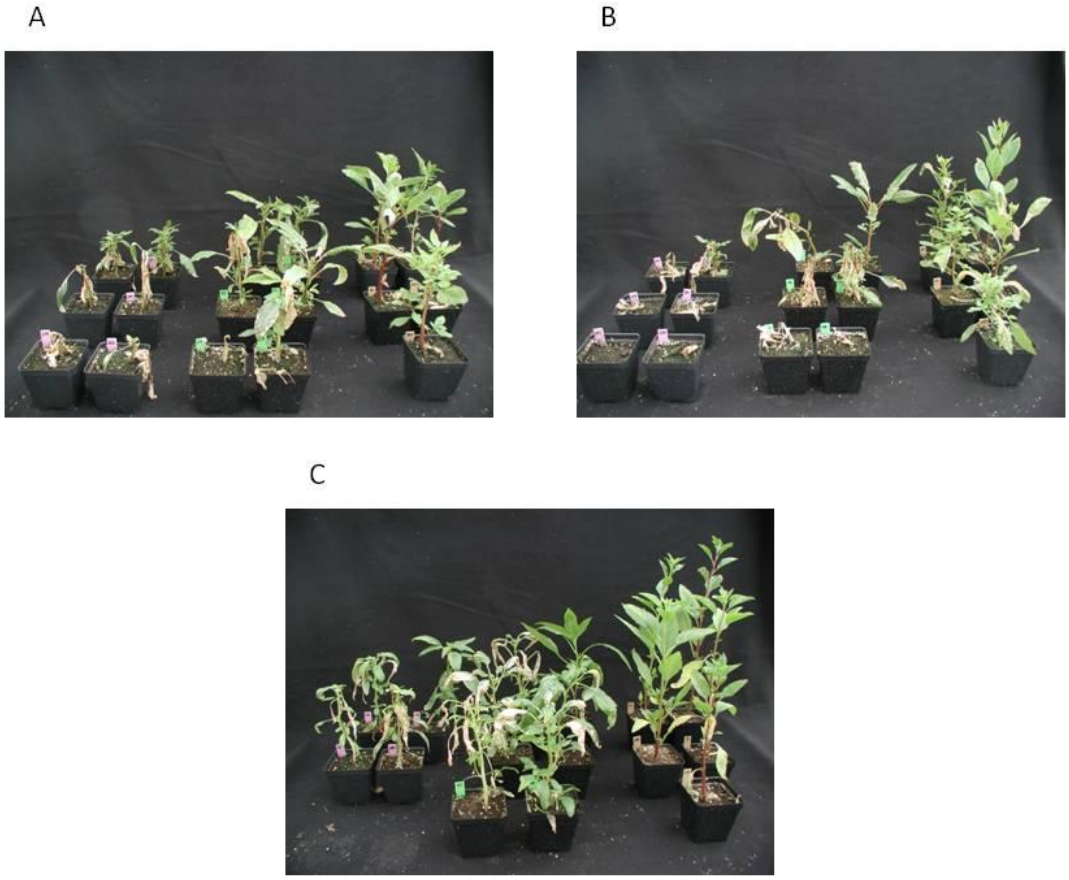

Figure 5.7 Pictures showing failure of multiple herbicides in consistent control of susceptible WCS plants. Biotypes shown are WCS, FCG, and ACR from left to right, and plants were treated with acifluorfen at 30 g ai ha^{-1} (A), with acifluorfen at 90 g ai ha^{-1} (B), or with atrazine at 200 g ai ha^{-1} (C). In each of the treatments, at least one WCS plant would have grown to produce seed, and therefore these treatments were not used in analysis of multiple resistance.

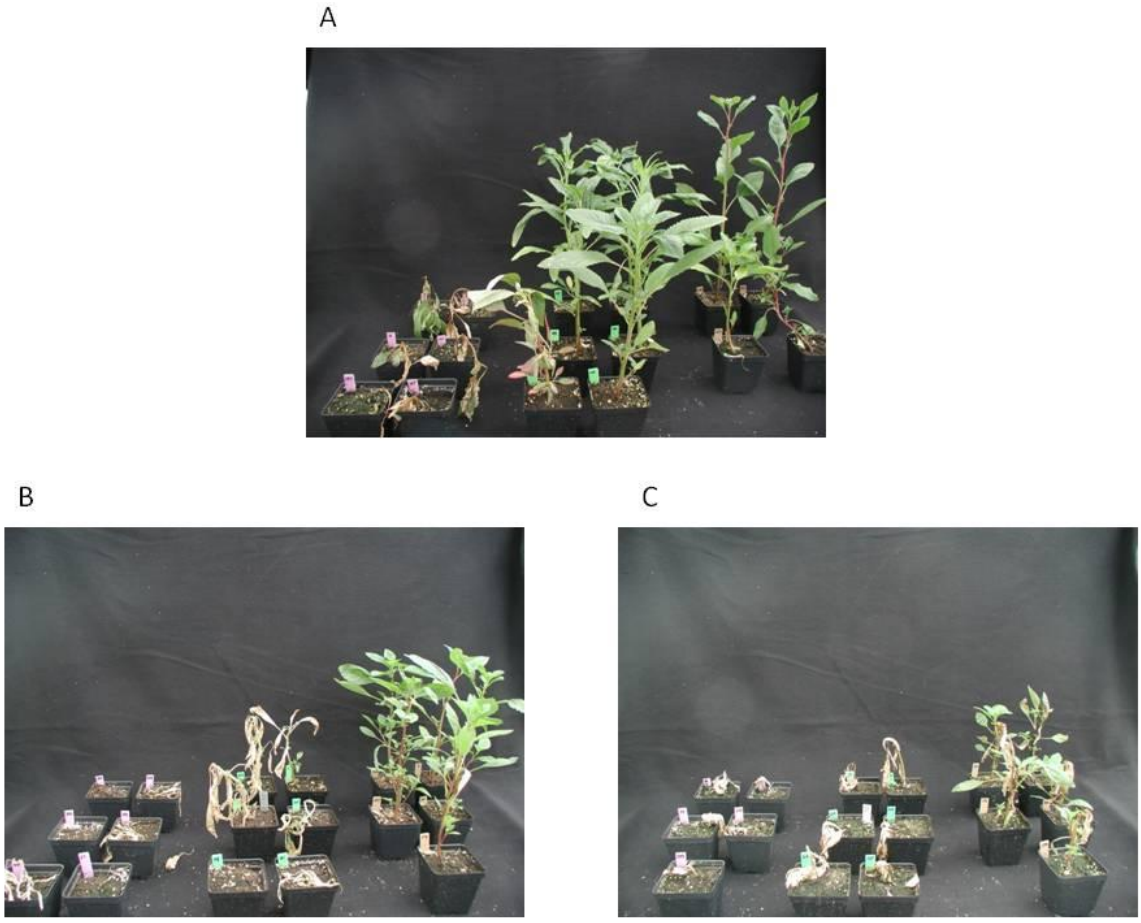

Figure 5.8 Pictures of the response of the FCG population to treatment with multiple herbicides at 16 DAT. The populations from left to right are WCS (the susceptible control), FCG, and ACR (the resistant control). Plants were treated with imazamox at 44 g ae ha^{-1} (A), atrazine at 1000 g ai ha^{-1} (B), and lactofen at 110 g ai ha^{-1} (C).

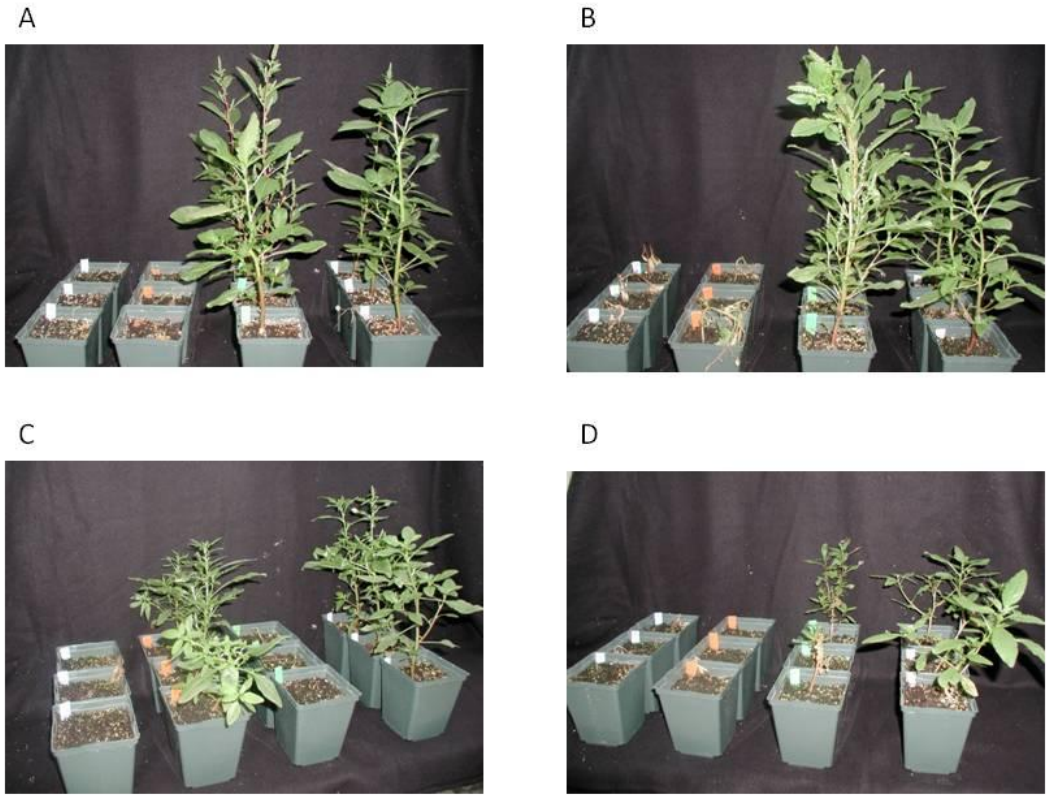

Figure 5.9 Pictures of the results of one run of the BCG multiple herbicide resistance screen. Biotypes from left to right are WCS (susceptible to all herbicides), MO1 (resistant to glyphosate), ACR (resistant to atrazine, imazamox and lactofen), and BCG. Plants were treated with atrazine at 1000 g ai ha^{-1} (A), imazamox at 44 g ai ha^{-1} (B), glyphosate at 3360 g ae ha^{-1} (C), and lactofen at 110 g ai ha^{-1} (D). The BCG population demonstrates resistance to all four herbicides.

Figure 5.10 Four-way resistant waterhemp individuals from a field population. Biotypes shown from left to right are WCS, MO1, ACR, and BCG (last 2 rows). Plants shown at the far right are BCG plants which survived a soil-applied treatment of imazethapyr at 1400 g ai ha^{-1} followed by (fb) atrazine at 1000 g ai ha^{-1} fb a mix consisting of glyphosate at 4200 g ae ha^{-1} and lactofen at 110 g ai ha^{-1}. Susceptible BCG plants were included in the 4th row from the left for the purpose of comparison with the four-way resistant individuals. The remaining plants shown were controlled by treatment with the glyphosate-lactofen mixture.

Table 5.1 Estimates of the glyphosate dose resulting in a 50% reduction in accumulated dry matter (GR_{50}) and resistance ratios for the FCG and MO1 populations when compared with the susceptible control, ACR. A four-parameter log logistic model was used to calculate the GR_{50} values in the first run, while a four-parameter, non-symmetric Weibull model was used to calculate the GR_{50} values in the second run, because the log logistic model failed to converge for run two. Runs are shown separately due to the presence of an interaction between runs. Values presented are mean estimates ± SEM.

	Dose Response Results for FCG Population			
	Run 1		Run 2	
Population	GR_{50}	R/S Ratio	GR_{50}	R/S Ratio
	g ae ha^{-1}		g ae ha^{-1}	
FCG	1400 ± 2800	25 ± 51	190 ± 87	1.7 ± 0.8
MO1	640 ± 630	12 ± 12	1700 ± 810	15 ± 7.4
ACR	55 ± 13	1.0	110 ± 5.2	1.0

Table 5.2 Estimates of the glyphosate dose resulting in a 50% reduction in accumulated dry matter (GR_{50}) and resistance ratios for the BCG and MO1 populations when compared with the susceptible control, ACR. A four-parameter log logistic model was used to calculate the GR_{50} values in both runs, which are shown separately due to the presences of an interaction between runs. Values presented are mean estimates ± SEM.

	Dose Response Results for the BCG Population			
	Run 1		Run 2	
Population	GR_{50}	R/S Ratio	GR_{50}	R/S Ratio
	g ae ha^{-1}		g ae ha^{-1}	
BCG	1400 ± 330	15 ± 7	1500 ± 480	3.1 ± 1.3
MO1	1200 ± 1100	13 ± 13	1600 ± 260	3.3 ± 1.0
ACR	95 ± 38	1.0	480 ± 130	1.0

Table 5.3 Response of the FCG waterhemp population to multiple herbicides and compared with that of the susceptible and resistant controls, WCS and ACR, respectively, recorded at 16 DAT with atrazine, lactofen, or imazamox. Data from two runs were pooled. Values presented are means ± SEM.

	Treatment[a]								
	Atrazine			Lactofen			Imazamox		
Biotype	% Surv[b]	% Ctrl[c]	Vis Rating[d,e]	% Surv	% Ctrl	Vis Rating	% Surv	% Ctrl	Vis Rating
WCS	0	4.2 ± 1.1	10 ± 0	0	5.6 ± 1.6	10 ± 0	0	15 ± 2	9.9 ± 0.1
FCG	5.9 ± 5.7	12 ± 3	9.2 ± 0.7	12 ± 8	7.5 ± 1.7	9.8 ± 0.2	82 ± 9	49 ± 9	6.8 ± 0.7
ACR	100	78 ± 6	0.5 ± 0.2	100	51 ± 6	1.3 ± 0.2	100	84 ± 8	0.8 ± 0.2

[a] Treatments consisted of atrazine at 1000 g ai ha^{-1}, lactofen at 110 g ai ha^{-1}, and imazamox at 44 g ai ha^{-1}.

[b] Percent survival was calculated by dividing the number of surviving plants by total number of plants.

[c] Mean percent of control dry weight was calculated by comparing the dry weight of each plant receiving a particular treatment with the mean dry weight of untreated plants of the same biotype.

[d] Visual ratings were assigned to each plant on a scale of 0 to 10, with a 0 indicating a plant that looked identical to untreated control plants of the same biotype, and a 10 indicating a dead plant.

[e] For percent survival and percent of control dry weight, n = 17 for FCG and ACR, and n = 18 for WCS. Visual ratings were only recorded in one run of the experiment, and these data are based on n = 12 plants for FCG and ACR, and n = 13 plants for WCS.

Table 5.4 Response of BCG waterhemp to multiple herbicides compared with that of susceptible and resistant controls, WCS and ACR, respectively, recorded at 16 days after treatment with atrazine, lactofen, or imazamox. Data from the first run are presented separately to show variable response to herbicides, attributed to use of a different seed pool in subsequent runs. Data from subsequent runs were pooled due to similar responses between runs. Values presented are means ± SEM.

	Treatment[a]									
	Atrazine			Lactofen			Imazamox			
Biotype	% Surv[b]	% Ctrl[c]	Vis Rating[d]	% Surv	% Ctrl	Vis Rating	% Surv	% Ctrl	Vis Rating	
WCS	0	6 ± 1	10 ± 0	0	7 ± 2	10 ± 0	0	13 ± 3	9.9 ± 0.1	
BCG	58 ± 14	39 ± 10	4.9 ± 1.3	83± 11	45 ± 7	3.2 ± 1.0	92 ± 8	77 ± 9	2.8 ± 0.9	Screen 1[e]
ACR	100	85 ± 6	0.5 ± 0.2	100	64 ± 6	1.3 ± 0.2	100	89 ± 9	0.8 ± 0.2	
WCS	0	0.7 ± 0.7		0	0.7 ± 0.7		0	6.6 ± 1.3		Subsequent Screens[f]
BCG	61 ± 5	59 ± 12		38 ± 7	10 ± 3		55 ± 11	65 ± 12		
ACR	100	90 ± 14		93 ± 7	7.1 ± 4.5		100	99 ± 13		

[a] Treatments consisted of atrazine at 1000 g ai ha^{-1}, lactofen at 110 g ai ha^{-1}, and imazamox at 44 g ai ha^{-1}.

[b] Percent survival was calculated by dividing the number of surviving plants by total number of plants.

[c] Mean percent of control dry weight was calculated by comparing the dry weight of each plant receiving a particular treatment with the mean dry weight of untreated plants of the same biotype.

[d] Visual ratings were assigned to each plant on a scale of 0 to 10, with a 0 indicating a plant that looked identical to untreated plants of the same biotype, and a 10 indicating a dead plant.

[e] In screen 1, n = 12 for all biotypes and treatments.

[f] In subsequent screens, visual ratings were not assigned. Percent control weights are based on n = 20 for BCG and n = 4 for WCS and ACR. Survival data are based on n = 80 BCG plants for atrazine, n = 50 BCG plants for lactofen, and n = 20 BCG plants for imazamox.

Table 5.5 Resistance mechanisms for FCG and BCG waterhemp populations.

Population	Resistance Type	Resistance Mechanism
FCG	ALS	W574L substitution in *ALS*
	PPO	ΔG210 deletion in *PPX2*
	Triazine	unknown
	Glyphosate	possibly due to gene amplification of *EPSPS*
BCG	ALS	W574L substitution in *ALS*
	PPO	ΔG210 deletion in *PPX2*
	Triazine	unknown; not due to known resistance-conferring target site mutation; possibly metabolism-based
	Glyphosate	unknown; not due to gene amplification of *EPSPS*; P106S mutation is present but does not cosegregate with resistance

CHAPTER 6

AN ATTEMPT TO TRANSFER GLYPHOSATE RESISTANCE FROM WATERHEMP TO SMOOTH PIGWEED

6.1 Abstract

Experiments were conducted to transfer glyphosate resistance from a confirmed glyphosate-resistant waterhemp (*Amaranthus tuberculatus*) population into smooth pigweed (*Amaranthus hybridus*). The potential for hybridization to occur between these species has been well-documented in previous studies. Resistance to acetolactate synthase-inhibiting herbicides has been successfully transferred from smooth pigweed to waterhemp, and with the recent evolution of glyphosate resistance in waterhemp, concern exists over the potential for interspecific gene flow to pass this resistance trait into other species. Numerous F_1 hybrids were obtained by crossing female waterhemp plants with smooth pigweed. F_1 plants were confirmed as hybrids by use of molecular markers in the internal transcribed spacer (ITS) region of ribosomal DNA. Hybrids were screened with glyphosate and found to be resistant. These plants were subsequently backcrossed to smooth pigweed to create BC_1 seeds. Due to low seed numbers, BC_1 plants were not screened for glyphosate resistance. These plants were grown until flowering and were again backcrossed to smooth pigweed to create BC_2 seed. Again very little seed was produced. The next step of this study will be to grow BC_2 plants through flowering to investigate segregation of reproductive systems. If monoecious plants are present, they should be self-pollinated, and their progeny should be screened for glyphosate resistance. If only dioecious BC_2 plants are present, these may be again backcrossed to smooth pigweed, but such a finding

would suggest that transferal of glyphosate resistance from waterhemp to smooth pigweed may be unlikely to occur in nature.

6.2 Introduction

Herbicides have become invaluable tools for weed control in agronomic cropping systems around the world. With the development of herbicide-resistant crops, chemical weed control has become even more convenient, as broad-spectrum herbicides can now be applied to crops to control weeds without damaging the crop. This convenience has led to the widespread use of such herbicides. At first glance this may seem like a positive step in the battle against weeds, but it has in fact led to a very negative consequence—namely herbicide resistance in weeds. Currently at least 194 weed species have evolved resistance to herbicides worldwide (Heap 2010), and current production practices are at least partially to blame for this epidemic, in that repeated application of a particular class or family of herbicides results in high selection pressure for resistance-conferring alleles in weed populations (Jasieniuk 1996).

However, the evolution of herbicide-resistance in weeds is not just a product of weed control practices, but is also dependent on genetic variation in weed populations (Jasieniuk 1996; Tranel et al. 2002). Genetic variation can be achieved through several mechanisms such as spontaneous genetic mutations, migration via seed or pollen movement, meiototic recombination, and interspecific gene flow, or hybridization (Jasieniuk 1996; Tranel et al. 2002; Trucco et al. 2005a).

The *Amaranthus* genus contains over 60 species (Hager et al. 2002), including both monoecious species, which primarily produce seed by self-pollination, as well as dioecious species in which plants are either male or female and thus must cross-pollinate to produce seed.

This genus contains several important weeds including waterhemp and smooth pigweed, both of which have become major problems in agronomic cropping systems in the Midwest.

Smooth pigweed is a monoecious small-seeded diploid (2n = 32) summer annual, which has long been a problem in the Midwest (Wax 1995; Hager et al. 2002), as well as around the world (Sauer 1967). Waterhemp is a diploid (2n = 32) small-seeded dioecious summer annual that is indigenous to North America (Sauer 1957), but this species has only become a major problem weed in the Midwestern United States within approximately the last 20 years (Hager et al. 2002). Both of these species have evolved resistance to photosystem II (PS II)-inhibiting herbicides as well as herbicides which inhibit acetolactate synthase (ALS) (Heap 2010). However, waterhemp has been even more problematic in that it has also evolved resistance to protoporphyrinogen oxidase (PPO)-inhibiting herbicides (Shoup et al. 2003) and glyphosate (Legleiter and Bradley 2008). Several factors are thought to be important in allowing these species to rapidly evolve resistance to herbicides.

Amaranths are known for their high seed production (Weaver et al. 1980; Sellers et al. 2003). In the case of waterhemp, the number of seeds produced may be in excess of 1×10^6 seeds per female plant (Steckel et al. 2003). This high seed production, when combined with meiotic recombination and spontaneous genetic mutations, necessarily implies that these species can produce genetically diverse progeny on which natural selection via herbicide application can then act to select herbicide-resistant individuals (Jasieniuk 1996; Tranel et al. 2002). Another factor which may be contributing to the success of these species in evolving herbicide resistance may be interspecific hybridization.

By as early as 1940 it was known that some species in *Amaranthus* can readily hybridize with one another (Murray 1940), and later studies proved that hybridization between *Amaranthus*

species can cause flow of herbicide-resistance genes from one species to another. In 1999 (a), Wetzel et al. demonstrated that ALS resistance could be transferred from *A. palmeri* to waterhemp through hybridization, and a study conducted by Fransen et al. (2001) confirmed that hybridization between these two species could indeed occur. In 2002, Tranel et al. reported success in transferring ALS-resistance from smooth pigweed to waterhemp through hybridization. Further, in 2005 (b) Trucco et al. reported that waterhemp and smooth pigweed may hybridize quite frequently under field conditions. These studies raised questions about the transferal of glyphosate-resistance between *Amaranthus* species.

Glyphosate, a broad-spectrum foliar-applied herbicide, is the most widely used herbicide in the world (Powles 2008). This is due in part to the commercialization of glyphosate-resistant crops beginning in 1996 with glyphosate-resistant soybean [*Glycine max* (L.) Merr.], which ultimately led to widespread use of this herbicide. In fact, by 2007 96% of soybean acres in the United States were planted with glyphosate-resistant varieties (Dill et al. 2008). This has contributed to 19 weed species evolving resistance to this herbicide worldwide, including waterhemp (Heap 2010). Glyphosate resistance in waterhemp has become a major concern for weed scientists and Midwest corn [*Zea mays* L.] and soybean producers alike, as this leaves few post-emergence options to control this weed—particularly in soybean.

The primary objective of this study was to attempt to transfer glyphosate resistance from waterhemp into smooth pigweed through hybridization between these species followed by backcrossing hybrids to smooth pigweed. Hybrid identification was aided by the results of previous work done by Wetzel et al. (1999b), in which internal transcribed spacer (ITS) molecular markers were used to identify *Amaranthus* species. Evidence is presented that

suggests that transferal of glyphosate resistance from waterhemp to smooth pigweed may be difficult to achieve in nature.

6.3 Materials and Methods

6.3.1 Plant culture

Unless otherwise indicated, all plants used in this study were grown from seeds sown in a 12 cm x 12 cm x 5 cm container in a medium consisting of a 3:1:1:1 mixture of commercial potting mix[1] to soil to peat to sand. When seedlings reached the two-leaf stage, they were transplanted into individual 6 cm x 4 cm x 5 cm inserts in 24 cm x 48 cm x 5 cm flats containing the previously mentioned growth medium. When plants reached 5 cm in height they were transplanted to 12 cm square pots containing 700 ml of growth medium, where they were allowed to grow until completion of the experiment. Plants were fertilized as needed using a slow-release complete fertilizer[2], and the plants were grown in the greenhouse under mercury halide and sodium vapor lamps that provided a minimum photon flux of 800 µmol m^{-2} s^{-1} at the plant canopy in addition to the light incident from the sun. The lamps were programmed for a 16-h photoperiod, and the greenhouse was maintained at temperatures of 22 C at night and 28 C during the day.

6.3.2 Herbicide application

All herbicide applications for this study were made using a compressed air, moving nozzle spray chamber with an adjustable platform and equipped with an 80015EVS even flat spray nozzle[3]. The nozzle was maintained at approximately 45 cm above the plant canopy. The

sprayer was calibrated to deliver 187 L ha^{-1} of water at 207 kPa. Plants were returned to the greenhouse immediately after spraying.

6.3.3 Selection of glyphosate-resistant waterhemp

The glyphosate-resistant waterhemp population used in this study was one from Missouri, designated as MO1, which was described previously (Legleiter and Bradley 2008). A waterhemp population from Adams County, Illinois, designated as Adams County Resistant (ACR) was used as a susceptible control. This population is resistant to PS II inhibitors, ALS inhibitors and PPO inhibitors, but is susceptible to glyphosate and also has been described previously (Patzoldt et al. 2005). Plants were grown as described above, until they reached 10–15 cm in height, at which time they were treated with herbicide. Glyphosate[4] was applied at rates of 840, 1680, 3360, and 6720 g ae ha^{-1} and treatments included 2.5% (v/v) ammonium sulfate[5] (AMS) and 0.25% (v/v) nonionic surfactant[6] (NIS). Five ACR and 24 MO1 plants were treated at each herbicide rate. An additional five plants of each biotype were kept as untreated controls. At two weeks after treatment, MO1 plants were rated as resistant (R) or susceptible (S) by comparison of their response to glyphosate with that of ACR. The multiple herbicide doses were used in order to ensure that at least one of the doses would allow for clear differentiation of R and S plants.

6.3.4 Creation of hybrids

After glyphosate-resistant MO1 plants were identified, they were allowed to grow until flowering began, at which time they could be identified as either male or female. Smooth pigweed [*Amaranthus hybridus* L.] (SP) seeds from a line susceptible to ALS-inhibiting herbicides, which has been previously described (Trucco et al. 2005a) were sown weekly and

grown as described above over the course of this experiment until glyphosate-resistant MO1 plants began flowering. This was done in order to ensure that flowering SP plants would be available as soon as the resistant MO1 plants began flowering.

When glyphosate-resistant MO1 plants began flowering, females (four in the first run and two in the second run) were selected for crossing with SP plants (Figure 6.1). These female plants were immediately moved into a separate greenhouse room isolated from male waterhemp pollen, and were placed in separate pollination bags[7] supported by ¾" PVC pipe, where they were allowed to continue to grow until they had branches containing numerous flowers, at which time a flowering SP plant was introduced into each of the pollination bags. In the first run of the experiment, crosses consisted of two tents each containing two MO1 females and one SP plant, and in the second run crosses consisted of two tents each containing one MO1 female and one SP plant.

Each morning after introduction of the SP plants into the pollination bags, the inflorescences of the waterhemp females were brushed against those of the SP plants to ensure that pollen transfer occurred. The pollen transfer was performed in the morning based on results of other studies, which indicate that some species in Amaranthaceae may release most of their pollen in the morning (Rodriguez et al. 2000; Singh and Babu 1980). SP and waterhemp plants were kept together in the pollination bags for at least three weeks, after which the female waterhemp plants were harvested and dried at room temperature for at least two weeks. F_1 seeds were then manually harvested and cleaned from the dried plants, keeping seeds from each female separate. The seeds were then stratified. The stratification procedure consisted of seeds first being surface sterilized by a 10 min treatment with a 1:1 mixture of bleach and water. Afterward,

the seeds were washed twice with sterilized deionized water, suspended in 0.15% (w/w) agarose, and then stored for at least two weeks at 4 C to break seed dormancy.

6.3.5 F_1 Glyphosate-resistance screen

Stratified seeds collected from the hybridization crosses were sown as described above, until they reached 10–15 cm in height, at which time they were treated with glyphosate at rates of 52.5, 105, 210, 420, 840, and 1680 g ae ha^{-1} in the first run and 840, 1680, and 3360 g ae ha^{-1} in the second run, where 840 g ae ha^{-1} corresponds to the usual field use rate. At least 10 plants from two of the four families created in the first run and from one of the two families created in the second run were screened in this way at each dose. Additionally, an untreated control group was included in both runs. The response of the putative hybrids to glyphosate was compared with that of the parental lines, SP and MO1, as well as the glyphosate-susceptible ACR population. The low rates were selected in the first run of the experiment because preliminary tests showed SP to be susceptible to glyphosate at rates as low as 52.5 g ae ha^{-1}, and hybrid plants were expected to demonstrate a level of glyphosate resistance between that of the parents based on the findings reported in Chapter 3 that glyphosate resistance is a partially dominant trait. At two weeks after treatment, hybrids were rated as either R or S by comparison with SP, MO1 and ACR, and plants appearing to be most resistant by visual inspection were selected for backcrossing to SP (12 plants in run one and 10 in run two). However, before the backcrossing was performed, molecular markers were used to confirm that the selected plants were indeed hybrids.

6.3.6 Hybrid confirmation

An approximately 100 mg sample of meristematic leaf tissue was collected from each of the selected glyphosate-resistant plants suspected to be hybrids as well as from several SP and MO1 plants. The tissue samples were placed in 1.5 ml centrifuge tubes[8] and kept on ice during collection. The samples were then stored at -80 C until needed.

Total DNA was extracted from meristematic leaf tissue by using a modified hexadecyltrimethyl-ammonium bromide (CTAB) protocol from Doyle and Doyle (1990), and the extracted DNA was resuspended in 50 µl of TE buffer. ITS regions one and two of each sample were then amplified in a polymerase chain reaction (PCR) using primers ITS4 and ITS5 described by Wetzel et al. (1999b). PCRs consisted of approximately 50–100 ng DNA, 0.2 mM of each dNTP[9], 1.5 mM $MgCl_2$, 0.8 µM of each of the forward and reverse primers[10], and 1.5 units of Taq polymerase[11] with a 1x concentration of supplied buffer in a final volume of 20 µL.

During PCR, the samples were subjected to an initial denaturation step of 94 C for 5 min, followed by 35 cycles of a denaturation step at 94 C for 1.5 min, an annealing step at 55 C for 1 min, and an extension step at 72 C for 1.5 min. After the final cycle, the samples were subjected to a final extension step at 72 C for 10 min, and were then stored at 4 C until analyzed.

Results of the PCR were analyzed via gel electrophoresis by running 5 µL of each product in a 1.5% (w/v) agaraose gel. Desired products were identified as bands on the gel corresponding to fragments of approximately 750 base pairs (bp) in length. The remaining 15 µL of PCR product in samples with successful amplification was then subjected to restriction enzyme digestion with *Hae*II as in Wetzel et al. (1999b), in order to determine whether the plants were actually hybrids.

Digestion reactions consisted of 0.2 µL BSA[12], 0.3 µL *Hae*II[13] (10 u μL^{-1}), 2.0 µL of the supplied 10X NEB4 buffer, 2.5 µL sterile deionized water, and 15 µL PCR product from ITS amplification, making a total reaction volume of 20 µL. Digestion reactions were allowed to proceed for at least 2 h at 37 C, after which time the products were investigated via gel electrophoresis by running them on a 1.5% (w/v) agarose gel at 80 V for approximately 2.5 h. *Hae*II was expected to cut the amplified fragment of the ITS region of SP, but not that of waterhemp. Hybrids were expected to show two bands on the gel, with one uncut band corresponding to the waterhemp copy and one cut band corresponding to the SP copy.

6.3.7 Creation of BC_1 lines

Confirmed hybrids were isolated from pollen sources once flowering began. They were allowed to grow in isolation for several weeks until the inflorescences were large, at which time they were pollinated with SP plants, in an attempt to transfer glyphosate-resistance back into SP (Figure 6.1). In the first run of the experiment the flowering hybrids were placed in a growth chamber set at 28 C during the day and 22 C at night and programmed for a 16-hour photoperiod. Meanwhile, multiple SP plants were allowed to flower in the greenhouse. Each morning SP inflorescences were shaken over a petri dish to collect pollen, and the pollen was then transported to the growth chamber where a small paintbrush was used to brush the pollen onto the inflorescences of the hybrids.

Based on the results of the first run of the experiment, in the second run the hybrids were placed in pollination bags in the greenhouse rather than in a growth chamber. When the inflorescences of the hybrids were large enough, SP plants were introduced into the pollination

bags with the hybrids. The SP plants were shaken each morning and SP inflorescences were brushed against hybrid inflorescences to transfer SP pollen to the hybrids.

SP plants were used to pollinate hybrids for at least one month, after which the hybrids were harvested and dried at room temperature for at least two weeks. BC_1 seeds were manually harvested from the hybrids. In the first run, this was done with a mechanical seed-cleaning device, while in the second run all hybrid flowers were ground by hand. Seeds were collected and stratified as described above.

6.3.8 BC_1 growth

Due to the small number of BC_1 seeds collected, the seeds were placed on moist filter paper and kept in an incubator at 35 C during the day and 30 C at night during germination in an attempt to successfully grow as many viable seeds as possible. Temperatures were alternated as previous studies have shown that this practice may improve germination rates in some *Amaranthus* species, including waterhemp and SP (Steckel et al. 2004). Germinated seeds were transported to the greenhouse and transplanted into 12 cm square 700 ml pots containing the previously mentioned growth medium.

Also due to the small number of seeds, the BC_1 plants were not screened with glyphosate. They were allowed to grow until flowering, at which time the plants were either again backcrossed to SP or allowed to self-pollinate in pollination bags, depending on whether the plants appeared to be dioecious (and female) or monoecious, respectively (Figure 6.1). Dioecious BC_1 plants were pollinated with SP as described above for at least one month after flowering began, and one monoecious BC_1 plant was placed in a separate pollination bag to isolate the plant from other pollen sources and was allowed to self-pollinate for at least one month. BC_1

plants were then harvested and dried at room temperature for at least two weeks, after which they were checked for seed production.

6.4 Results and Discussion

6.4.1 General observations on putative hybrids

To maximize the number of hybrids obtained from the initial crosses between waterhemp and smooth pigweed, waterhemp was used as the female plant, as in Tranel et al. (2002). This was done because smooth pigweed is a monoecious plant and is therefore predisposed to self-pollination (Trucco et al. 2005b). Thus, had the crosses been performed in the other direction (i.e., a glyphosate-resistant male waterhemp plant crossed with smooth pigweed), much of the seed collected from the smooth pigweed plant would likely be the result of self-pollination of the smooth pigweed plant rather than the result of the desired hybridization. By using waterhemp as the female, any seed collected would most likely be hybrid seed ignoring any potential apomixis, which has been shown to be of minimal importance in waterhemp (Bell and Tranel 2010).

Waterhemp was found to hybridize readily with SP, as has been reported previously (Tranel et al. 2002, Trucco et al. 2005b), with in excess of 1000 F_1 seeds being produced by each of the waterhemp females used in such crosses (data not shown). Germination of the F_1 seeds was similar to that of seeds from the parental populations. After germination, however, the hybrid seedlings did not appear as healthy as seedlings of MO1 and smooth pigweed at the same growth stage. Hybrid seedlings often exhibited small lesions or necrotic spots on the leaves, and in a significant number of hybrid seedlings (up to half of those that germinated in some cases) whole leaves eventually became necrotic, resulting in plant death either before or during the intial transplanting from the 12 cm x 12 cm x 5 cm containers into the 6 cm x 4 cm x 5 cm

containers. While not severe enough to kill all of the putative hybrid plants, these spots were not apparent on the MO1 and SP seedlings, which may indicate that a fitness penalty is associated with hybridization between these two species.

Eventually the F_1s that survived the initial transplant began to look just as healthy as MO1 and SP plants at the same growth stage. By the time F_1s reached 10–15 cm in height, they displayed morphological characteristics that fell between those of the parental populations—most noticeably in the leaves, which was similar to the observations made by Tranel et al. (2002). The leaf shape of the hybrids was like that of smooth pigweed at the base, but the tips generally came to a point, creating a triangular-shaped leaf which could often be distinguished from that of either smooth pigweed or waterhemp. The leaves were similar to those of waterhemp in that they were hairless. The leaves sometimes displayed purple spots, which were not visible on either smooth pigweed or waterhemp. However, while hybrids could usually be identified by visual inspection in the greenhouse, it is likely that such plants would be much more difficult to identify in the wild, due to the common morphological similarities among species within the *Amaranthus* genus (Wetzel et al. 1999b; Sauer 1955; Costea et al. 2001; Hager et al. 1997), and phenotypic plasticity that may be caused by environmental factors (Trucco 2005d).

6.4.2 Transferal of glyphosate resistance to F_1s

Resistance to glyphosate was successfully transferred from waterhemp into the F_1 populations as evinced by screening the F_1s with glyphosate. In the first run of the experiment F_1s survived glyphosate up to 1680 g ae ha^{-1} (the highest rate applied), which effectively controlled all SP and ACR plants, allowing for selection of glyphosate-resistant F_1s. In the

second run of the experiment F_1s survived glyphosate up to 3360 g ae ha^{-1}, which was the highest rate applied in this run, and both the 1680 and 3360 g ae ha^{-1} rates effectively controlled all SP and ACR plants, again allowing for selection of glyphosate-resistant F_1s. However, although the plants survived treatment with glyphosate and generally looked different from either of the parental populations, there remained some uncertainty as to whether these plants were actually hybrids, and so tests were conducted to eliminate this uncertainty by using molecular markers to confirm plants as hybrids.

6.4.3 Molecular confirmation of hybrids

F_1 plants were easily identifiable as hybrids via the molecular methods described by Wetzel et al. (1999b). After digestion with *Hae*II, waterhemp samples showed a single uncut intense band at approximately 750 bp, while SP showed a faint uncut band at 750 bp as well as a more intense cut band at approximately 650 bp (Figure 6.2). The faint 750 bp band present in digested SP samples is attributed to incomplete digestion of the amplified ITS region by the restriction enzyme. Because DNA from hybrid plants should contain both the waterhemp and the SP ITS regions, two bands were also expected after digestion of these samples. However, the double bands of hybrid plant samples were distinguished from the double bands of the smooth pigweed samples by the fact that in the hybrid samples the intense band occurred at 750 bp and the faint band occurred at 650 bp—the opposite of the SP bands. The expectations for hybrids were two bands of roughly the same intensity. The fact that the 750 bp band is more intentse is most likely due at least in part to the incomplete digestion which was obvious in the SP samples, although larger bands in general are more intense than shorter bands. All putative hybrids for

which successful amplification and digestion of ITS occurred were confirmed as hybrids. Plants were discarded if amplification of their ITS regions failed.

6.4.4 Creation of BC_1s

When F_1 plants reached maturity and began to flower, all plants were found to be dioecious, as has been observed in other studies in which monoecious species have been crossed with dioecious species where the dioecious species serves as the maternal parent (Murray 1940; Tranel et al. 2002; Trucco et al. 2006). All of these plants appeared to be female, although stigmas were rarely visible.

As has been documented previously, fertility in the F_1 plants was greatly reduced compared with that of the parental populations (Murray 1940; Tranel et al. 2002; Trucco et al. 2006). In fact, fertility level was so low in the F_1 generation that complications in the first run of the experiment led to a decision being made to start over from the beginning. A mechanical seed-cleaning device was used to collect and clean seeds from hybrid plants in the first run, and a total of approximately 50 seeds were collected from 8 plants. These seeds were germinated on filter paper as described above, and those that germinated (approximately 20) were planted in soil. However, as the seedlings began to grow, nearly half of the plants were identified as common lambsquarters [*Chenopodium album* L.], indicating contamination during seed cleaning. As the seed cleaning device had been used to clean *Chenopodium* as well as waterhemp seeds in the past, and because of the high frequency of *Chenopodium* seeds present in the seed lots collected from the F_1s, there could be very little confidence that the non-*Chenopodium* seedlings were actually BC_1 seedlings, so all remaining seeds were discarded, and the experiment was repeated.

Several techniques were modified in the second run of the experiment, as described in the Materials and Methods. First, the pollination technique was modified in an attempt to collect more seeds from the F_1s. The amount of light in the growth chamber seemed to be less than ideal for growth of the plants, so in the second run the F_1s were grown in pollination bags in the greenhouse. Also of concern was that the best time to collect viable pollen from smooth pigweed was unknown, as was the amount of time that pollen remained viable after falling from the smooth pigweed plant. It seemed possible that pollen viability could have been reduced by collecting it from smooth pigweed in a petri dish and transporting it to the F_1 plants in a growth chamber, so in this run of the experiment smooth pigweed plants were placed inside the pollination bags with the F_1s.

Perhaps most importantly, however, was that in the second run of the experiment great care was taken to prevent contamination of BC_1 seed lots. This time flowers from F_1 plants were ground by hand in the lab over white paper to aid in identifying seeds, and seeds that fell onto the paper were collected with forceps and placed in 1.5 ml microcentrifuge tubes. A total of approximately 100 seeds were collected from 7 plants in this run.

6.4.5 Observations on BC_1s

Out of the 100 BC_1 seeds collected, 55 germinated and were grown in the greenhouse. Due to the fact that the seeds germinated over an extended period of time (at least two weeks), as well as the small number of seeds collected, the plants were not screened for glyphosate resistance, as indicated in the Materials and Methods. The hope was that these plants would segregate for mono- and dioecism. The monoecious plants would then be selfed and the seed collected from such plants would be grown and screened for glyphosate resistance. However,

once flowering began all but one of the 55 BC_1 plants grown appeared to be dioecious and female, just like the F_1s. One plant appeared to be monoecious, as a few anthers were visible on this plant, so it was allowed to self pollinate. The other BC_1 plants were kept in pollination bags and backcrossed again to smooth pigweed as was done with the F_1 plants.

Fertility in the BC_1 plants overall was very low, similar to that of F_1 plants. In fact, 30 of the 55 BC_1 plants in this study produced no seed (Figure 6.3). One seed was collected from the plant that had appeared to be monoecious. However, 6 plants did produce > 10 seeds per plant, with 1 of these plants producing approximately 50 seeds. These findings are similar to those of Trucco et al. (2005c) and Tranel et al. 2002, in which variability in seed production of BC_1 plants was observed. However, in the previous studies, a few of the BC_1 plants were observed to produce more than 1,000 seeds—a finding which was not supported by the results of this experiment. Perhaps this could be due to a difference in pot size, as Tranel et al. (2002) used 1.4 L pots and Trucco et al. (2005c) used 4 L pots, while 0.7 L pots were used in the current study. Presumably, using larger pots would allow for more plant growth, including higher flower production, which could in turn lead to a higher seed production. It is interesting to note, however, that frequencies of plants producing 0 seeds were similar to that observed by Trucco et al. (2005c).

6.4.6 Future work

It remains yet to be determined whether glyphosate resistance may be successfully transferred from waterhemp to smooth pigweed. The next step of this study will be to plant the BC_2 seed and grow the plants to investigate segregation in reproductive systems. Due to low seed numbers, this generation should again be grown to flowering without being screened with

glyphosate. When flowering begins, any monoecious plants that are observed should be self-pollinated to produce seed. Assuming significant seed production occurs in such plants, the progeny should then be screened for glyphosate resistance by comparison with resistant MO1 and susceptible smooth pigweed. If such plants are found to be resistant to glyphosate, then the transferal of glyphosate resistance may be deemed a success.

If no monoecious plants are observed in the BC_2 progeny, the study could potentially be continued by again backcrossing dioecious plants to smooth pigweed to create BC_3 seed. However, if this step is required, it may be argued that transferal of glyphosate resistance from waterhemp to smooth pigweed will be fairly unlikely to occur in nature, due to the observed difficulty in obtaining seed from hybrid and BC progeny even when pollen is brushed directly onto the flowers of such plants in the greenhouse. Presumably, this direct pollen transfer would be less likely to occur in a field setting, although it is possible, providing that hybrid or BC plants are growing in close proximity to smooth pigweed plants. Also, if three or more generations of backcrossing to smooth pigweed are required to transfer glyphosate resistance into this species, the transferal process could easily be interrupted in a field in which a farmer practices herbicide rotation. Such a practice could potentially easily kill hybrid or BC plants containing the glyphosate-resistance genes before they are able to be transferred into smooth pigweed.

Thus, the results of this study indicate that transferal of glyphosate resistance from waterhemp to smooth pigweed is quite difficult to achieve in the greenhouse, and so it is probably unlikely to occur soon in nature. This is not to say that it is impossible. Nature has a way of achieving things that initially may seem very unlikely or perhaps even impossible. Multiple studies have shown the high frequency at which hybrids can be formed by waterhemp females being pollinated by smooth pigweed. In fields where both species grow in close

proximity and plants survive to produce seeds, some hybrids are likely to be found growing alongside waterhemp and smooth pigweed, despite a possible fitness penalty in the hybrid plants. It has been shown that these hybrids are capable of producing a small number of seeds when pollinated by smooth pigweed (Trucco et al. 2006), but on a whole-field level the total number of BC_1 seeds produced by hybrids may be quite large. This would lead to an increased chance of naturally-occurring BC_1 plants again being pollinated by smooth pigweed to produce seeds, and after yet another one or two generations of backcrossing, it may be possible for smooth pigweed to acquire glyphosate resistance from waterhemp.

6.5 Sources of Materials

[1] LC1 professional growing mix, Sun Gro Horticulture Canada Ltd., 52130 RR 65, P.O. Box 189, Seba Beach, AB 70E 2BO Canada. Distributed by Sun Gro Horticulture Distribution Inc. 15831 N.E. 8th St., Suite 100, Bellevue, WA USA 98008.

[2] Scotts Osmocote Classic 13-13-13 slow-release fertilizer. The Scotts Company LLC, 14111 Scottslawn Rd., Marysville, OH 43041.

[3] TeeJet 80015EVS spray nozzle. TeeJet Technologies, P.O. Box 7900, Wheaton, IL 60187.

[4] MON 76255 40.2% ae Technical Grade Glyphosate. Monsanto Company, 800 N. Lindbergh Blvd., St. Louis, MO 63167.

[5] N-PaK® AMS Liquid, Winfield Solutions, LLC, P.O. Box 64589, St. Paul, MN 55164-0589.

[6] Activator 90 Nonionic Surfactant. Loveland Products, Inc. PO Box 1286, Greeley, CO 80632.

[7] 78” x 72” 1.75 mil Pollination Bags. Vilutis & Co., Inc. 1135 Center Rd., Frankfort, IL 60423.

[8] Seal-Rite 1.5 ml Microcentrifuge Tubes. USA Scientific, Inc., PO Box 3565, Ocala, FL 34478-3565.

[9] Invitrogen 100 mM dNTP Set, PCR Grade, Invitrogen Corporation, 5791 Van Allen Way, P.O. Box 6482, Carlsbad, CA 92008.

[10] IDT Custom Oligos. Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241.

[11] *Taq* DNA Polymerase. Invitrogen Corporation, 5791 Van Allen Way, P.O. Box 6482, Carlsbad, CA 92008.

[12] Purified BSA 100x. New England Biolabs, 240 County Road, Ipswich, MA 01938-2723.

[13] *Hae*II Restriction Enzyme. New England Biolabs, 240 County Road, Ipswich, MA 01938-2723.

6.6 Acknowledgements

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6.8 Figures

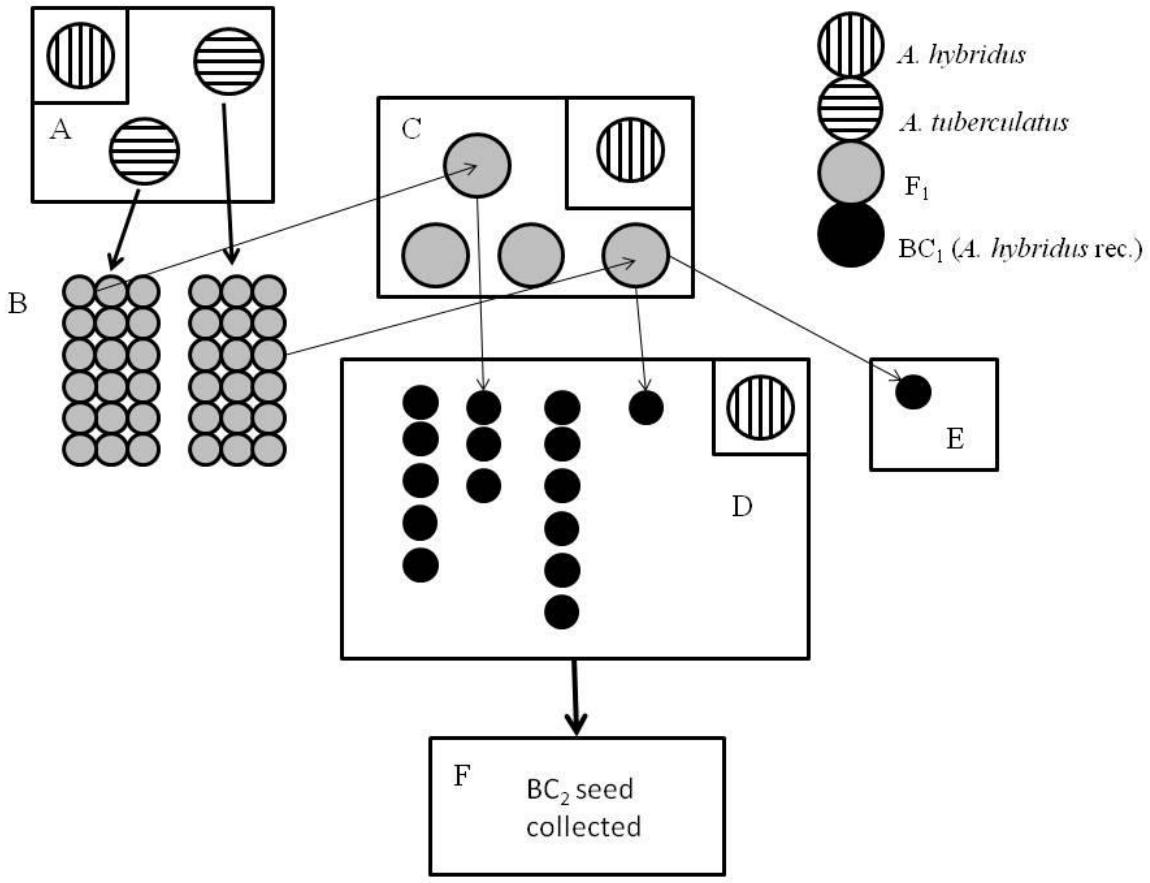

Figure 6.1 Pedigree of BC_2s. Some arrows indicating lineage have been removed from the figure to improve clarity. (A) Initial hybridization of *Amaranthus hybridus* and *Amaranthus tuberculatus* (circles represent plants). (B) Herbicide selection step to select glyphosate-resistant progeny. (C) Glyphosate-resistant F_1 females backcrossed to *Amaranthus hybrids*, the recurrent parent. (D) BC_1 females backcrossed to *Amaranthus hybridus*. (E) A monoecious BC_1 plant, which was selfed. (F) Few seeds were produced by the BC_1 plants.

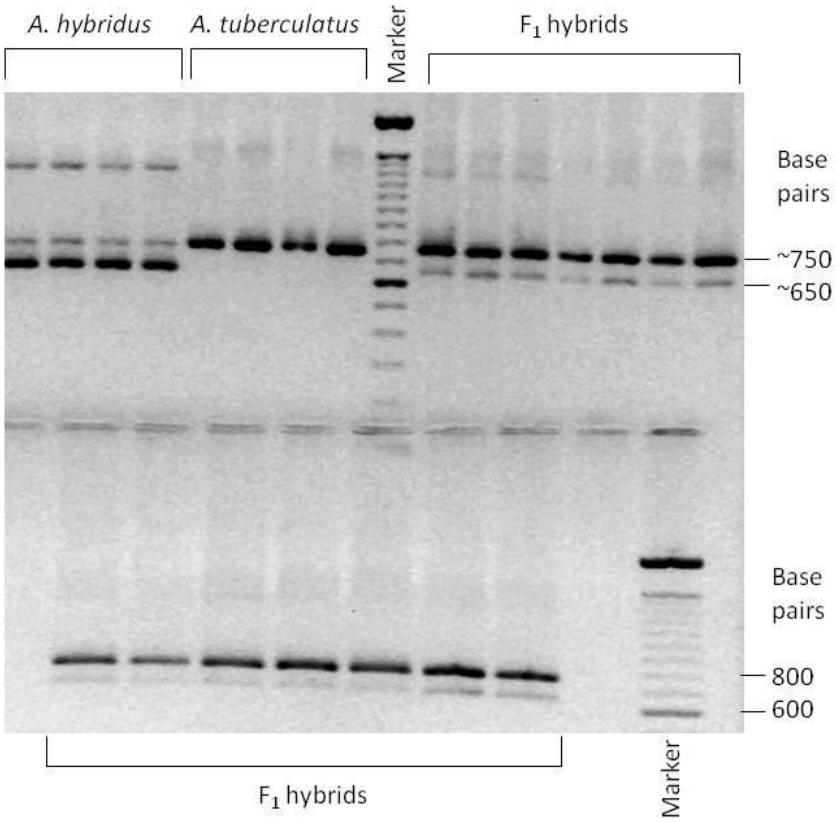

Figure 6.2 Confirmation of hybrids by use of molecular markers. The ITS regions of *A. hybridus*, *A. tuberculatus*, and F_1 hybrids were amplified and digested with *Hae*II. Only the digested products are shown. All *A. hybridus* ITS regions were cut, while all *A. tuberculatus* ITS regions remained uncut by the enzyme. The faint band at 750 bp in *A. hybridus* samples is attributed to incomplete digestion. F_1 hybrids tested show two bands—the faint band at 650 bp is from the *A. hybridus* copy, while the intense band at 750 bp is from the *A. tuberculatus* copy as well as incomplete digestion of the *A. hybridus* copy.

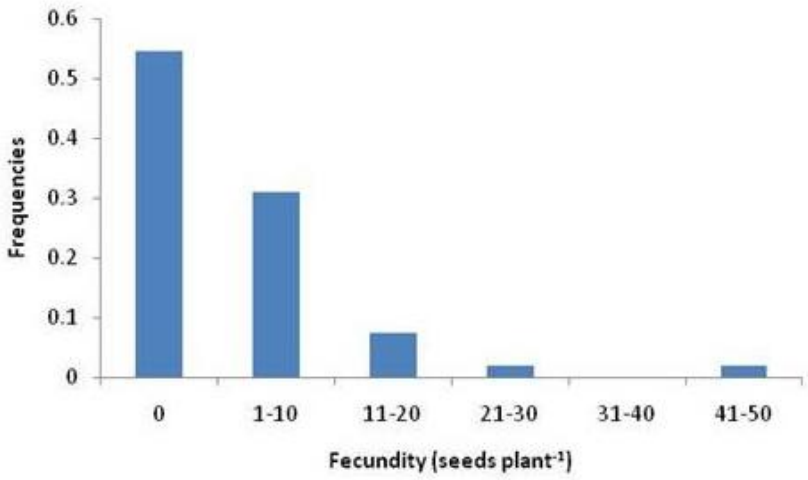

Figure 6.3 Distribution of fecundities of BC_1 plants. Frequencies represent number of plants with seed production falling into the respective fecundity category divided by the total number of 55 BC_1 plants grown in the study.

CHAPTER 7

CONCLUDING REMARKS

7.1 Conclusions, Implications, and Future Directions

Weed control has come a long way since humans first began farming in approximately 10,000–15,000 B.C (Wells 1961). From perhaps originally not even recognizing the harmful effects of weeds on crops, to simply "putting up" with weeds, to mechanical control through hand-weeding and the use of ever more specialized tillage implements, to the eventual widespread use of effective herbicides for chemical weed control, humans have made outstanding achievements in this field, with some of the most significant coming mainly within the last 100 years (Timmons 1970). With adapting weed control practices, however, have come adapting weed species, many of which have evolved resistance to various herbicides in the past several decades (Heap 2010).

One of the worst weeds in Illinois is waterhemp (Wax 1995; Hager et al. 2002), known for its high seed production (Steckel et al 2003; Sellers et al. 2003), C_4 photosynthesis (Costea et al. 2005), prolonged germination period (Hartzler et al. 1999), and ability to rapidly evolve resistance to herbicides (Tranel and Trucco 2009). Over the past two decades, waterhemp has been observed to evolve resistance to herbicides of four different modes of action (Heap 2010), and this weed is quickly becoming something of a nightmare to Midwest farmers—particularly those attempting to control this weed with postemergence herbicides in soybean. To date, at least two populations of waterhemp have evolved resistance to three of the four available herbicide options for postemergence chemical weed control in soybean (PPO inhibitors, ALS inhibitors and glyphosate) (Chapter 5; Legleiter and Bradley 2008), leaving glufosinate as the only

alternative. With a potential increase in reliance on glufosinate for future postemergence waterhemp control, it will be a matter of time before waterhemp evolves resistance to this herbicide as well.

From seed biology, to the genetics of herbicide resistance, to the creation of individual waterhemp plants resistant to four different herbicide modes of action, even to an attempt to transfer herbicide resistance from this waterhemp to another related species, this thesis is the product of four years of work on myriad aspects of this successful weed species. At first glance, these topics may seem better-suited to be discussed separately, in the theses of two or more students rather than just one. After all, what does seed production have to do with herbicide resistance anyhow? However, at the heart of this thesis ultimately was an investigation into factors that make this species a successful weed, and this is the glue that ties these perhaps seemingly disjointed studies into a cohesive whole.

This thesis began with, in Chapter 2, a study on seed biology of waterhemp. Several studies have been conducted on the amount of seed production in waterhemp (Sellers et al. 2003; Steckel et al. 2003), dormancy and germination of waterhemp seeds (Steckel et al. 2004; Leon et al. 2007), and persistence of waterhemp seeds in the soil seed bank (Buhler and Hartzler 2001; Steckel et al. 2007), but this was the first that addressed the time required for seed production after pollination in this species. The results of the study on seed maturation indicate that waterhemp seeds may become viable in as little as 7 to 9 days after pollination (DAP) when dried after harvesting or in as little as 12 DAP if chilled after harvesting, and that the level of dormancy in these seeds decreases when they remain on the plant for at least 15–30 days. These results have several implications, one of which is related to the success of waterhemp as a weed. After a waterhemp female is pollinated, it need only survive for approximately one week to

produce viable seeds, after which time, whatever happens to the female plant is of little importance, as the seeds already have been produced. This fact makes waterhemp well-suited for seed production in rapidly changing environments. One example could be growth on riverbanks, where short gestation time is desirable due to the somewhat unpredictable nature of flooding, which could potentially kill plants of longer gestation time after pollination but before seed production occurs. Another situation where short gestation time may be beneficial is in agronomic cropping systems—particularly in soybean. It is not uncommon for frequent rainfall to delay the application of postemergence herbicides in soybean in mid- to late-June, which is near the time at which some of the earliest flowering begins in waterhemp. Thus, if waterhemp plants begin flowering approximately one week before a farmer is able to apply herbicide to his field, even if the herbicide kills the female waterhemp plant, it may already be too late to prevent seed production.

The decrease in dormancy levels of waterhemp seeds that remain on the plant for 15–30 days after pollination may be another beneficial trait of this species. Because waterhemp displays an indeterminate growth pattern (Costea and Tardif 2005), the plants continue to produce seeds until they are killed by frost or other means. The observed high levels of dormancy in newly developed seeds may serve to postpone the germination of such seeds produced near the end of the growing season until the following season. However, if instead, dormancy levels were low in newly developed seeds, there would be a better chance that these seeds would germinate during a warm spell in the fall and the resulting seedlings would likely be killed by frost before being able to produce new seeds.

Another implication of this study is that it demonstrates the speed with which generation advancement can occur in waterhemp, which is particularly applicable to weed scientists

working with this species for genetics studies. If level of seed production is not a concern, then viable seed can be collected from females used in crosses within 14 DAP (this is a conservative estimate—viable seed would likely be available for use within 7–9 DAP) if the seeds are dried at room temperature and then stratified. If the seeds must be stored at low temperatures, the results of this study indicate that it is best to wait until at least 20 DAP before harvesting in order to obtain the highest germination percentages. A potential weakness of this study is that it involved only one population of waterhemp. It is conceivable that the amount of time required to produce viable seeds after pollination may vary from one population to another, and thus it would be interesting to perform this same experiment on plants from other populations for comparison.

In Chapter 3, the genetics of glyphosate resistance in a waterhemp population from Missouri was discussed. Several of the impetuses for conducting this study were to better predict the spread of glyphosate resistance in waterhemp, to develop strategies to slow the evolution of glyphosate resistance, and even to perhaps discover a novel mechanism of resistance to glyphosate—the most widely used herbicide in the world (Preston and Wakelin 2008).

In the beginning of this study, the hypothesis was that glyphosate resistance is conferred by a single nuclear-inherited dominant gene, as are most other types of reported herbicide resistance (Jasieniuk et al. 1996). The results of the study indicate that glyphosate resistance is a nuclear inherited trait, which was determined through the observation of glyphosate-resistant individuals present in all lines of F_1 progeny derived from reciprocal crosses performed between resistant and susceptible individuals. The trait appears to be partially dominant based on the levels of resistance in the F_1 progeny. Segregation for glyphosate resistance was observed in every F_1 line, although the amount of segregation varied by family, as well as by glyphosate dose. Analysis of segregation in backcrossed lines (to susceptible) as well as F_2 lines did not

always fit expectations for a single-gene trait, making the number of genes responsible for conferring resistance unclear. During this experiment, glyphosate resistance in Palmer amaranth (a related species) was shown to be due to an elevated copy number of *EPSPS* (Gaines et al. 2010). When waterhemp was tested for the presence of this same mechanism, it was indeed found that resistant plants from the Missouri population contained a 3 to 5-fold increase in *EPSPS* copy number over that of susceptible plants. However, when copy number was examined in F_1 plants in relation to level of glyphosate resistance, elevated copy number seemed to be, at best, weakly correlated with resistance. In the F_2 plants it seemed that increased copy number may have been even less correlated with resistance level, with resistance and copy number seeming to segregate almost independently of one another. This indicates that, if gene amplification is involved in conferring resistance to glyphosate in waterhemp, it is definitely not the only factor responsible. This is supported by the observation of no elevated *EPSPS* copy number in another glyphosate-resistant waterhemp population from Illinois (Chapter 5).

Thus, the number of genes responsible for conferring glyphosate resistance, as well as the mechanism conferring glyphosate resistance in this population, is yet unknown. However, the conclusions that can be drawn from this study may still have some implications. Because the resistance is nuclear-inherited, it can spread through pollen flow, so rapid spread of glyphosate resistance to neighboring fields or regions is expected. The fact that the resistance is partially dominant implies that resistance can spread even more rapidly, for only one resistance-conferring allele need be present in a plant for that plant to survive treatment with glyphosate. This in contrast with a recessive resistance trait, such as resistance to trifluralin in green foxtail [*Setaria viridis* (L.) Beauv.] (Jasieniuk et al. 1994), which would spread much more slowly in an outcrossing species such as waterhemp, because two recessive alleles must be present in a plant

in order for it to survive herbicide treatment (Jasieniuk et al. 1996). Finally, the segregation for inheritance of copy numbers of *EPSPS,* particularly in the F_1s, suggests that the multiple copies of this gene present in the Missouri population are likely located in multiple regions of the genome as was found by Gaines et al. (2010) to be the case in Palmer amaranth. Southern blot analysis of individuals in the MO1 population could potentially shed light on whether or not this assertion is true.

In Chapter 4, it was shown that four types of herbicide resistance could be combined into a single plant relatively easily by crossing individuals from ACR (a population resistant to PS II inhibitors, PPO inhibitors and ALS inhibitors) with individuals from the glyphosate-resistant Missouri population described in Chapter 3. To show this, individuals were screened with a soil-applied ALS inhibitor followed by either a sequential treatment with a PS II inhibitor followed by a mixture of glyphosate and a PPO inhibitor, or by simultaneous application of a PS II inhibitor, a PPO inhibitor, and glyphosate. Observations on control treatments applied to test the efficacy of these three herbicides in combination with one another provided some interesting information, showing that little if any antagonism occurred with simultaneous application of the herbicides. As a cautionary statement, however, it should be mentioned that the glyphosate rate in such mixtures was at five times the regular field use rate, while lactofen (the PPO inhibitor) was applied at only one-half the regular field use rate, and atrazine (the PS II inhibitor) was applied at the usual field use rate. It is unknown whether using a lower rate of glyphosate would have produced the same no-antagonism effects that were observed in this study.

Linkage studies conducted on one particular F_2 line derived from such crosses indicated tight linkage between ALS resistance and PPO resistance, as nearly every plant found to contain ALS resistance was also found to contain PPO resistance, despite the fact that by screening

plants from this line with either one of these two herbicides revealed that only approximately two thirds of the F_2 individuals were ALS- or PPO-resistant. Because these two resistances are known to be conferred by two separate mechanisms (via dominant mutations in single genes), it is likely that the genes containing the resistance-conferring mutations (*ALS* and *PPX2*) are located near one another on the same chromosome.

After this demonstration of the possibility of combining resistances to herbicides with four different modes of action into a single plant, Chapter 5 provided evidence of the discovery of a four-way resistant waterhemp population in the field. More specifically, two Illinois waterhemp populations were investigated for multiple herbicide resistance. One of these populations was found to contain a high frequency of ALS resistance and to segregate for glyphosate resistance, with the resistant individuals displaying phenotypes similar to those of resistant plants from the Missouri population. A second Illinois population was also tested for multiple resistance, and it was in fact found to contain resistance to glyphosate, ALS inhibitors, PPO inhibitors, and PS II inhibitors. Further studies proved that individuals in this four-way resistant population were themselves four-way resistant plants, thus confirming the assumed implications of creating such a four-way resistant population in the greenhouse—that it could be expected to evolve in the field as well. This is the first reported four-way resistant waterhemp population. The first multiple resistance reported in waterhemp was by Foes et al. (1998) of a population resistant to ALS inhibitors and triazines. Three-way resistance was reported in a waterhemp population (ACR) by Patzoldt et al. (2005), which was found resistant to ALS and PPO inhibitors and triazines. Now that a four-way resistant waterhemp population has been discovered, it seems likely that four-way resistance will spread, which would severely limit options for postemergence herbicidal control of this species in soybean. In fact, without the

utilization of soil-applied herbicides, such as dinitroanilines (e.g. trifluralin) and chloroacetamides (e.g. metolachlor), there soon may be no chemical control options left for this species in soybean.

Another interesting aspect of this study was the investigation into resistance mechanisms of both populations. Both populations demonstrated mutations known to confer ALS resistance (W574L in *ALS*) and PPO resistance (ΔG210) in other waterhemp populations. The mechanism of triazine resistance was not discovered, but target-site resistance was ruled out, perhaps suggesting metabolism-based resistance as the mechanism. As for glyphosate resistance, the population resistant to only glyphosate and ALS inhibitors has been shown in other studies to contain elevated copy number of *EPSPS* (Liu, Riggins, Tranel unpublished), which may be involved in conferring glyphosate resistance in this population. However, the other population, which was tested for elevated copy number in this study, was found to show no *EPSPS* gene amplification. Sequencing *EPSPS* in this population did reveal a mutation (P106S) shown to confer moderate levels of glyphosate resistance in other species (Jasieniuk et al. 2008; Baerson et al. 2002), although this mutation did not cosegregate with resistance in this population. In fact all plants sampled (6 of them) from this population showed this mutation. While this discovery did not lead to the identification of the glyphosate-resistance mechanism in this population, it did suggest that this mutation may serve as a genetic marker, allowing for the tracking of gene flow in inheritance studies of glyphosate resistance as in hybridization experiments.

In Chapter 6, an attempt was made to transfer glyphosate resistance from waterhemp to smooth pigweed. Previous studies have shown that these two species frequently hybridize with one another under field conditions (Trucco et al. 2004, 2005a). This was found to be the case in the greenhouse as well, with numerous seeds collected from glyphosate-resistant waterhemp

females that were crossed with smooth pigweed. Crosses were performed in this way to ensure a high frequency of hybrids. In theory, since waterhemp is a dioecious plant, all seeds collected from a waterhemp female in such a cross should be the result of hybridization. Hybrids were confirmed by the use of ITS markers and all suspected hybrids proved to be true hybrids when subjected to such testing. The hybrids displayed a glyphosate-resistant phenotype when screened with herbicide. Interestingly, this also lends proof to the conclusion made in Chapter 3 that glyphosate resistance is a partially dominant trait.

Glyphosate-resistant hybrids (which were all dioecious and female) were backcrossed to smooth pigweed in order to transfer the resistance back into this species. However, the F_1 hybrids produced very few seeds from such crosses and thus the progeny were not screened for glyphosate resistance. Instead, the progeny were grown and observed for monoecism, and out of 55 plants, one appeared to be monoecious. This plant was allowed to self-pollinate, while the remaining plants were again backcrossed to smooth pigweed, again resulting in very low seed production. In future work, these seeds should be sown and observed for monoecism. In the case that monoecious plants are discovered, they should be allowed to self-pollinate and the progeny should be screened for glyphosate resistance. In the case that no monoecious plants are found, these plants could again be backcrossed to smooth pigweed in hope of obtaining a higher number of seed. However, if this additional backcross is required, it would seem quite unlikely that glyphosate resistance can be successfully transferred from waterhemp to smooth pigweed in nature.

An interesting additional experiment to perform in the (unlikely) case of successful transfer of glyphosate resistance from waterhemp to smooth pigweed (showing that gene introgression in this direction of hybridization is in fact possible) would be to attempt to transfer

ALS resistance from the synthetic four-way resistant waterhemp population described in Chapter 4 into smooth pigweed. Assuming successful transfer of this trait, it seems likely that PPO resistance would also be successfully transferred into the species simultaneously, due to the linkage between these resistances that was reported in Chapter 4. However, based on the results of the work performed for Chapter 6, as well as a report of the failure to observe waterhemp *ALS* in monoecious plants derived from hybridization between waterhemp and smooth pigweed (Trucco et al. 2005b), it seems unlikely that successful transferal of *ALS* resistance into smooth pigweed would be achieved.

Waterhemp truly is a fascinating weed—at least as far as weed scientists are concerned. One of the most interesting aspects of this weed is that it has only recently emerged from behind the scenes, and it is now one of the most difficult weeds to control in soybean fields in the Midwest due to its rapid evolution of herbicide resistance. In the preceding pages are contained numerous examples of the many problems that this troublesome weed can pose for agronomic production. In the future this weed will, no doubt, continue to be useful in exemplifying what it means to be a successful weed, both irritating farmers and intriguing weed scientists along the way. It is the hope of the author that future weed scientists will find the information contained in this thesis useful, and that they will be able to build upon the framework which has herein been laid out.

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APPENDIX A

A PRACTICAL GROWER'S GUIDE TO EXPERIMENTATION ON WATERHEMP

A.1 Introduction

The preceding thesis is the fruit of the past four years that I have spent conducting experiments on waterhemp. Presumably, in every fruit can be found a seed, which allows the species to last for another generation. The following is a seed which has been extracted from the fruit of the thesis with the hope of providing useful information for the next generation of weed scientists studying waterhemp at the University of Illinois.

For the novice and expert alike, waterhemp can be quite finicky to grow in a greenhouse. It is common for the grower to encounter problems such as little or no germination of seeds after planting, a lack of uniformity in plant heights at the time of treatment with herbicide, an unexpected failure of herbicide to control known susceptible plants, and a prolonged flower initiation phase, among others. All of these may have serious negative consequences on the outcome of an experiment, in some cases necessitating the repetition of a study. This treatise is meant to draw attention to common pitfalls that a novice may unwittingly stumble into, and to aid the reader in ascension of the learning curve to growing well-behaved waterhemp plants, thereby saving the student both time and effort.

A.2 Stratification

A.2.1 Why bother?

Breaking seed dormancy is essential to achieving uniform plant height (i.e. uniform experimental replicates) in any experiment involving waterhemp. The level of dormancy varies

from population to population, although most of the populations with which I have dealt, such as ACR and MO1, will germinate fairly uniformly after one month of stratification, with ACR demonstrating uniform germination after as little as two weeks of stratification. Others—most notably WCS—will rarely germinate uniformly even after being stratified for months, although stratification certainly does improve germination of seeds of this population. I recommend stratifying WCS seeds for at least six months to achieve the best germination. Because of the large time requirement for stratification of these seeds, it is recommended that many WCS seeds be stratified immediately upon joining the lab, and that the student then continue to stratify WCS seeds as they are used, being sure to maintain a stock of seeds that have been stratified for at least six months.

After stratification, it is time to begin the experiment. At this point, in deciding how many seeds to plant it is helpful to have an idea of the expectations for germination percentages of sown seeds of various biotypes. For populations such as MO1 and ACR, approximately 50–80% of stratified seeds germinate within 48–72 hours after sowing. For stratified WCS seeds, one may expect a delay in germination by at least 24–48 hours compared with ACR and MO1, and that, at best, approximately 50% of these seeds will germinate together, with continued germination of the remaining seeds lasting for at least another week.

In cases in which time prohibits the luxury of stratification, one may still achieve adequate germination by planting more seeds than would usually be necessary. If waterhemp seeds must be sown without having been stratified, one may expect a prolonged germination period, with only approximately 30% or fewer of the sown seeds germinating within the 48–72 hour range for ACR and MO1, and perhaps only 10% of WCS seeds germinating within one week of planting. Therefore, sowing WCS seeds without stratification is not recommended, as

one will observe meager stand counts even after sowing many more seeds than would usually be necessary.

A.2.2 Okay, stratification is important. So how do I do it?

Before stratification, a proper container should be selected in which to store seeds during stratification. In the past, I have used 1.5 ml microcentrifuge (Eppendorf) tubes, as this generally provides enough seeds for at least two plantings, or perhaps more if few seeds are needed for a particular experiment. When one has a large supply of seeds to work with, it may be tempting to fill these tubes nearly to the top with seeds. However, during the stratification procedure discussed below, one will quickly find that this is a mistake, as this leads to pipetting problems and seed loss. These issues can be avoided by resisting the urge to fill the eppendorf tubes with seeds. It is best to fill the tubes only up to the 0.5 mL mark, and if more seeds are needed, then more tubes of the same seed type should be prepared. This will allow for space in the tubes for the liquids used during the stratification process.

The tried and true method for stratification that I have always used is to first soak the seeds in a 1:1 mixture of bleach and water for 10 min. If the seeds are being stratified in 1.5 mL tubes, a good volume to use is 500 µL each of water and bleach, being sure to add the water first to prevent causing damage to the seeds, which may occur if they are soaked in undiluted bleach. This bleach treatment step is important in sterilizing the surface of the seeds, particularly in the case of WCS, which will need be stored for at least six months at 4 C. Without sterilization, mold may begin to grow on the seeds, and from past experience this situation should be avoided, as germination of moldy seeds can be expected to be at nearly 0%. Thus the bleach treatment of seeds before stratification is critical—especially for long-term storage. Although not investigated

to my knowledge, it is thought that the bleach treatment may also play a role in helping to break dormancy, perhaps by partially degrading the seed coat, which is yet another reason to treat seeds with bleach before stratification.

After addition of the bleach/water solution, the tube should be capped and shaken to ensure that all seeds are coated with the solution, as the solution tends to stay on top of the seeds otherwise. After allowing seeds to soak in the bleach/water solution for ten minutes, this solution should then be pipetted from the seeds (using a 1000 µl pipette with the appropriate tips). When doing this it is important not to push the pipette tip too firmly into the seeds, or it may become clogged, and a new tip will have to be used. It is common for seeds to stick to the pipette tip during withdrawal from the tube. The amount of seed loss can be lowered by wiping excess seeds from the pipette tip to the rim of the tube, although some seed loss will probably still occur. This is to be expected, and should not be a cause for concern. Pipette tips should be discarded in a biohazard bag (which should later be autoclaved) if they have been used for seeds of herbicide resistant biotypes. Although the same tip may be used for multiple tubes of the same type of seeds, the student should be careful to make sure that the tips are changed between tubes of different biotypes to prevent contamination of the seed lots.

After removing the bleach/water solution from the tubes, the seeds should be rinsed twice with sterile water. That the water is sterile is essential. If non-sterilized water is used, this may potentially negate the effect of the bleach treatment, which could lead to mold growth on the seeds during stratification. The water that I use for stratification is deionized water taken from the Barnstead EASYpure LF machine (Figure A.1), although any type of sterile water should be acceptable. This machine should be run for approximately 30 seconds before turning on the flow of water, or until the display reads at least 17.0 MΩ-cm. The water should be collected in a

bottle, and the bottle should then be loosely capped and autoclaved under a liquid cycle for 20–30 min. Afterward, when the water has cooled, the bottle should be labeled as "Sterile H_2O" and can then be used for stratification, as well as for any other procedure requiring the use of sterilized water.

To rinse the seeds, I add the same volume of sterilized water as was added of the bleach/water solution (1000 µl of water if 1.5 ml tubes are being used). After addition of the water, the tubes should be capped and shaken to ensure adequate contact between water and seeds in order to remove bleach from the seed coats. In the case of seeds having no other plant material such as pericarps in the tube, this water can then be immediately extracted with a pipette in the same way that the bleach/water solution was removed. However, if other plant material such as pericarps or stem fragments are present in the tubes with the seeds, it may be wise to let the seeds soak in the water for several minutes to allow the residual bleach to seep out of such porous materials. Once this water is removed, the seeds should be rinsed a final time with the same volume of water as was used for the first rinse (although the exact volume is not critical so long as all seeds can be submerged). Once this water has been removed, it is time for the final step of the stratification procedure, which is the addition of agarose to the tubes.

Agarose is used in stratification for two purposes. One is that the agarose contains water and thus allows the seeds to become imbibed during stratification—a step that must occur before germination can proceed—thus helping to speed the germination process after sowing. The other reason for using agarose is due to its viscous nature and is important during the seed sowing process. If the seeds are stored in water, they tend largely to sink to the bottom, making the planting process a sort of "all or nothing" event, as it is difficult to select only the number of seeds desired for planting under such circumstances. However, if the seeds are stored in agarose,

they can be suspended throughout the solution by vigorous shaking, which then allows the grower to be more selective in pipetting out the desired number of seeds during the planting process.

Again it is important that this agarose be sterilized in order to take full advantage of the seed surface sterilization provided by the bleach treatment. Based on my experience, I have found that a 0.1–0.15% (w/v) agarose solution works well for seed suspension. I generally make the agarose in 100 ml aliquots. This requires the measurement of 0.1 – 0.15 g of agarose to be dissolved in 100 ml of water. Here it is not necessary that the water be sterilized (although the use of sterilized water is quite acceptable), as the agarose powder may not be sterile and thus will need to be autoclaved anyway. Therefore, any type of water may be used. 100 ml should be poured in a bottle containing the agarose powder, and the bottle should then be loosely capped and autoclaved under a liquid cycle for 20–30 min. It is important that the agarose be allowed to cool before use in stratification, as the addition of hot agarose may cause damage to the seeds. Therefore, it is best to plan ahead for stratification to ensure that cooled and sterilized agarose is available when needed for stratification.

If stratification is being performed in 1.5 ml tubes, approximately 1000 µl of sterilized agarose should be added to the rinsed seeds. The tubes should then be capped and vigorously shaken to suspend the seeds, and then should be stored at 4 C until needed. It is helpful for the student to write the stratification date on the tubes as well as his or her initials and the type of seeds contained in the tube, as, although it may not seem possible early on in the research project, things can become quite confusing later without adequate labeling of the tubes. This comes from four years of personal experience, and the student would be wise to pay heed to this advice.

After two weeks, one month, or perhaps six months of stratification, depending on the biotype, the seeds are finally ready to be sown, marking the true beginning of the experiment. However, before sowing the seeds, the grower should be well-informed on issues pertaining to soil.

A.3 Considerations on Soil

A.3.1 What kind should I use?

The soil mixture that has been used by myself and other members of the Tranel lab in the past is a 3:1:1:1 mixture of commercial potting mix : soil : peat : sand (or 1:1 LC1: weed mix) (Figure A.2). This tends to be better than using either LC1 alone or weed mix alone. Plants grown in LC1 tend to be starved for nutrients—much more so than plants growing in weed mix. However, weed mix is a much heavier soil than is LC1 which may potentially cause unnecessary injury to seedlings during transplanting. Another disadvantage to using pure weed mix is that this soil tends to retain less water than does LC1, thus requiring more frequent watering. Therefore, we have found that a 1:1 mixture of these two soil types is ideal for waterhemp growth in the greenhouse.

A.3.2 I need to start a project, but how much soil will I need?

In my experience it has been somewhat difficult to accurately estimate the amount of soil I will need for a particular project, for whatever the reason. However, I have come up with some guidelines which generally seem to come somewhat close in predicting soil use requirements. For the usual pots used by members of the Tranel lab (4.5” square 700 mL pots), one may expect to fill approximately 400 pots per small soil cart (Figure A.3). Thus, a large soil cart (twice the

size of a small cart) will fill approximately 800 of these pots. If 4” standard (round) pots are used, one may expect to fill approximately 800 of these pots with a small soil cart. It is best to order enough soil to keep some extra for other smaller projects that may arise, such as seed planting or transplanting into flats. Communication is critical at this stage (see discussion on communication below). It is important to know whether labmates are planning to use soil in the near future, and if so, the amount that they plan to use. It is also proper etiquette to inform labmates if the grower feels that he or she will come close to using all of the remaining soil, so that labmates will have time to order soil for themselves before it becomes too late to do so.

A.3.3 When should I order my soil?

The greenhouse staff requests that they be notified at least one week in advance of the date that the soil is needed. It is sometimes difficult to accurately predict when soil will be needed (most often due to miscommunication or lack of communication among labmates). Therefore, the importance of communication should again be stressed. In the past I have always tried to allow at least one week between when soil is ordered and when it will be needed. Oftentimes the soil will still be delivered earlier than expected. It is very rarely delivered later than expected. However, even when attempting to follow these guidelines, occasional emergencies will undoubtedly arise. In such cases, the importance of maintaining a positive relationship with members of the greenhouse staff (discussed below) can be quite beneficial. I myself have experienced several emergencies (due to my own fault of not discussing soil needs with my labmates), in which I discovered that no soil was available when I needed the volume of a large soil cart by the next day. In these cases, due to my friendly relationships with the greenhouse staff, they were able to make an exception and mix my soil immediately, having it

delivered the following day. However, the grower should strive to avoid these situations at all costs.

A.3.4 What should I do with the soil when I'm done?

After soil in the newly-delivered soil cart has served its purpose, any remaining soil should promptly be transferred to one of the white plastic soil tubs (Figure A.4) labeled for the Tranel lab so that the soil cart may be used to deliver soil to other greenhouse users as soon as it is necessary. As an added benefit, doing this will help to keep the grower's relationship with greenhouse staff a positive one.

A.4 Seed Sowing

A.4.1 How should I plant my seeds?

The technique used to sow waterhemp seeds will depend somewhat on the individual and what he or she finds to work best. I have found that often the easiest technique (particularly with seeds that have been stratified) is to use a pipette. When preparing for seed planting, the grower should take all supplies that might be necessary to prevent having to make extra trips between the greenhouse and the lab (which will take a minimum of 10 minute each). Thus, when I went to the greenhouse to plant seeds, I would take with me a 1000 µL pipette, a box of the appropriate pipette tips, scissors, a bottle of 0.1%–0.15% agarose, a biohazard bag, plastic tags, a pencil or a marker, a lab notebook, and all of the necessary seeds. (Using a box to carry these supplies is recommended.)

However, before planting, some preparatory work is required. The amount of time required to finish this work depends on the condition of the soil. Before planting, it is necessary

that containers have been filled with soil, and that the soil has been moistened. For seed sowing, I usually used flats without holes and 08-01 plastic inserts (Figure A.5, Figure A.6). If the soil stock is moist, then simply wetting the soil surface thoroughly before planting will suffice. However, if the soil stock has been allowed to become dry, it is likely that wetting the soil in the 08-01 inserts will not have the desired effect of dampening the soil throughout. In fact, most of the water will sit on the surface of the soil or run over the sides of the container. In this case, it is best to use the 08-01 inserts in flats with no holes. Inserts should be filled with soil as shown in Figure A.6. The flats should then be filled with water, and the soil allowed to soak overnight. By the next day, the soil should be damp, and excess water can be poured from the flats. The soil is then ready for use.

After the initial preparations have been made, it is time to sow the seeds. To do this, place a tip on the 1000 µL pipette. Then, using the scissors, cut off the end of the tip approximately half-way between the narrowest point and the widest point of the tip, perhaps cutting slightly closer to the narrow end. This will allow for the pipette tip to be used to extract seeds from the tubes in which they were stratified (see section A.2.2). After cutting off the tip, set the pipette to approximately 500 µL (or slightly less). The tubes should be vigorously shaken to resuspend the seeds in the agarose. Then use the pipette to extract the desired number of seeds, and expel the seeds from the pipette tip directly onto the damp soil surface in the desired insert, making an attempt to distribute the seeds as much as possible.

In some cases it is difficult to select the desired number of seeds in a single pipette extraction, although most of the agarose may have been already extracted. In such cases, more seeds may be extracted by pipetting more agarose from the bottle into the seed-containing tube. The width of the opening on the cut pipette tip may also have to be adjusted to allow for more

seeds to enter the tip. It is important to prevent contamination of the seed lots by both seeds from other lots, as well as by unsterile pipette tips. Particularly if a pipette tip has touched the soil, it should be discarded to keep the agarose sterile and to allow for continued long term storage of unused seeds without fungus growth in the tubes. All pipette tips should be discarded in a biohazard bag to prevent the release of seeds of herbicide-resistant waterhemp populations into the wild. It is not uncommon to experience difficulties during seed sowing, and it will be left to the grower to determine the precise sowing method most suitable to his or her style. Seeds of different populations should be sown in inserts distributed in a checkerboard fashion (Figure A.6) to prevent or minimize contamination among seed lots.

Immediately after sowing seeds of a particular population, a pencil or a marker should be used to mark on a plastic tag the name of the population sown, the date, and the grower's initials to prevent possible subsequent confusion on which populations have been sown in which containers and when they were sown, among other potential sources of confusion. After sowing is complete, a watering can should be used to lightly water the soil surface on which the seeds have been sown. This serves several purposes, including 1) re-moistening the soil to aid with germination, 2) lightly burying the seeds to allow them to stay moist for longer periods of time, and 3) distributing the seeds across the soil surface. The third purpose is very important, as seedlings do not grow uniformly when many seeds germinate in a small clump. After watering the soil, the supplies should be taken back to the lab, and the remaining seeds placed back into storage at 4 C.

A.4.2 Ok, I know how to do it, but how many seeds should I plant?

Based on the discussion in the stratification section, the number of seeds planted depends on the number of plants needed for the experiment, as well as on the biotype of the particular seeds being planted. Another important factor is whether or not the seeds have been stratified for the recommended amount of time. For ACR seeds stratified for at least 2 weeks, MO1 seeds stratified for at least one month, or WCS seeds stratified for at least 6 months, one may expect 50–80% of the ACR and MO1 seeds to germinate within 2–3 days, and one may expect approximately 50% of the WCS seeds to germinate within 3–5 days. For unstratified seeds, one may expect, at best, 30% of ACR and MO1 seeds to germinate within the first 48–72 hours after planting. For unstratified WCS, likely only 10% or fewer of the seeds will germinate within the first week of being sown.

Another factor to consider when deciding how many seeds to plant is that uniformity in plant size is desirable when conducting an experiment, but even waterhemp plants that germinate at the same time may show a range of sizes at the time of treatment. Therefore, it is recommended that extra plants be grown so that non-uniform plants may be discarded before treatment. I suggest planting enough seeds to obtain at least twice as many plants as necessary at the beginning of an experiment.

A.5 Seedling Care

A.5.1 I have planted my seeds. Now, how should I care for them?

After sowing seeds, it is critical that the soil surface be kept moist—and this is especially true immediately after germination, at which time the root system has not developed and the plants are very susceptible to death due to dry soil conditions. This should be done gently with a

watering can to prevent flushing seeds over the edges of the inserts. This is a good time to use liquid fertilizer on the seedlings (see Appendix C), which can be mixed in with the water in the can. If a request must be made for watering by the greenhouse staff over a weekend when the grower will be out of town, it is recommended that the grower first instruct a member of the greenhouse staff (or leave a note near the seedlings) on how to properly water the seedlings. Otherwise, it is not uncommon for a grower to come back to the greenhouse on a Monday only to discover that many of the seedlings have been killed due to high-pressure watering from the hose.

A.5.2 Soon my seedlings will be too crowded. What should I do about it?

Usually by 48–72 hours after sowing, some evidence of seed germination is obvious. The seedlings usually display 1–2 true leaves by 12–14 days after sowing. At this stage, in the interest of uniformity in plant sizes, I generally transplant seedlings to 08-06 inserts in flats with holes (Figure A.5). It is important that this transplanting be done as soon as most of the seedlings exhibit 1–2 true leaves, or else competition among the seedlings will soon lead to very uneven plant heights as well as plant stress (discussed later). The inserts I usually use consist of 48 wells per flat.

Before transplanting, it is critical that the soil in the 48-well inserts be very moist. Once this soil has been thoroughly watered, the grower can proceed with transplanting. To do this, I generally poke holes in each of the 48-well inserts with my index finger, inserting it up to the second joint. Then I gently pull a seedling from the 08-01 inserts out of the soil (sometimes loosening the surrounding soil with a plastic tag is helpful), taking care to leave most of the roots intact. The extracted seedling may then be placed into one of the holes in the 48-well inserts.

During transplanting, the grower should attempt to choose uniform seedlings. This is the first level of selection to perform in ensuring uniformity at the time of treatment, and obvious differences in growth stage of seedlings at this age tend to become more pronounced later in the experiment. So, by spending a bit of extra time during the initial transplanting, the grower can prevent having to discard as many plants later.

At this stage, if feasible, I recommend transplanting at least 50% more plants than will be needed in the experiment. However, in large experiments this could require an unreasonable amount of extra time, and in such cases, I recommend transplanting at least 10–25% more plants than will ultimately be needed.

Although it may be tempting to leave much of the seedling stem exposed above the soil during transplanting, I have discovered that this is a mistake. The seedlings are still quite fragile at this stage, and if they are allowed to rise several centimeters above the soil surface after transplanting, they will likely fall over and be buried during subsequent watering. Thus, I usually transplant seedlings so that only approximately the top 1 cm of the plant remains exposed above the soil surface. I then close the hole by gently pushing soil against the stem. After transplanting 48 (or the fewer if a whole 48 are not needed) seedlings, I water the flat with a watering can to aid the roots in growing into the new soil. The grower should be sure to use plastic tags to mark which populations have been transplanted to avoid potential confusion later. Once transplanting has been finished, the seedlings may be allowed to grow for approximately one more week before a final transplant becomes necessary.

A.5.3 When should I transplant to pots?

When plants in the 08-06 inserts have grown to approximately 5 cm in height, it is time to transplant them once more—this time into pots. This usually occurs approximately 7 days after the initial transplanting. From my experience, the best pots to use for studies involving herbicide application are the 4.5" square 700 mL pots (as opposed to smaller pots). Presumably the extra root growth allowed by use of such pots causes plants to experience less stress than they would experience when growing in smaller pots, and therefore the plants should display a more favorable response to the herbicide (i.e. the plants used as susceptible controls should be killed by the herbicide). These pots are not kept in stock and must be ordered by sending a request to a member of the greenhouse staff. The pots are referred to by the manufacturer as KORDLOK SQL0450 pots.

To transplant to pots, I have found that the fastest way (and nearly the only way when an experiment consists of 800 plants or more) to transplant is to first fill the pots with soil (I usually fill 48 pots at a time, corresponding to the number of plants growing in a flat containing an 08-06 insert) nearly to the top. If the soil in the flats is damp, then the soil in the pots need not be watered prior to transplanting. I then draw together four fingers on one hand and insert them into the soil in the center of a pot, making a hole approximately finger-length deep. With fingers still in the soil, I then rock my fingers forward and backward to increase the size of the opening. Finally, I detach one of the 08-06 inserts, invert it, and, gently pushing on the bottom of the insert, remove one of the six plants contained in the insert. I then place the plant, with the soil plug still intact, into the hole made in the center of the pot and gently push the plant deeper into the soil. It is important that this is done gently. By pushing too firmly, the grower may break

some of the roots extending horizontally just below the soil surface. When this happens, the plants generally begin to wilt within minutes and will tend to fall onto their sides.

This stage of transplanting marks the second selection opportunity for ensuring uniformity in plants at the time of treatment. If plants are obviously above or below the average height at this time, or if they look unhealthy or otherwise irregular for whatever reason, they should not be transplanted to pots. However, at this stage it is recommended that the grower keep approximately 10% more plants than will be treated to allow for a final round of selection for uniformity at the time of treatment. After transplanting is complete (or more often, if desired) the pots should be watered thoroughly several times to allow the roots to begin growing into the new soil contained in the pots.

A.6 Fertilizer

A.6.1 Should I fertilize my plants?

Without fertilizer, waterhemp plants will become quite visibly stressed. The stems become wiry, and the leaves thick and waxy—and the leaf area remains quite low compared with fertilized plants, which amounts to differential amounts of herbicide uptake between fertilized and unfertilized plants. Eventually the leaves will even turn yellow. As mentioned earlier, stressed plants often do not respond favorably to herbicide treatment. In fact, known susceptible plants will often survive treatment with herbicide under sufficient levels of stress. Therefore, although fertilizer may seem relatively unimportant and may be easily forgotten by the grower, it is highly recommended that the grower apply fertilizer to his or her plants. This can often mean the difference between the success or failure (and necessary repetition) of an experiment.

A.6.2 I know I should fertilize my plants, but how?

As mentioned in the seedling care section, the easiest way to fertilize seedlings is to apply a liquid fertilizer in combination with water from a watering can. This may be more beneficial than application of a solid fertilizer at this stage, as roots are not widely distributed through the soil, and presumably, with the application of liquid fertilizer, some nutrient uptake may occur through plant leaves. During seedling growth before the initial transplant, approximately two such applications of liquid fertilizer should be sufficient, with one additional application perhaps being made after the initial transplant but before the plants have been transplanted to pots.

During transplanting to pots is when I usually begin applying solid fertilizer to plants. Although I have not experimented with different types of fertilizer, I have found that 13-13-13 osmocote works well. (See Figure A.5 for a demonstration on how to record the use of fertilizer taken from the greenhouse storeroom.) I generally apply ½ Tbsp. of fertilizer to each pot after the initial watering. Obviously, however, the amount added depends upon the size of the pot being used. And I have never observed negative effects on the growth of a waterhemp plant due to too much fertilizer. After addition of the fertilizer to the pots it is important to use care when watering (which can now be done with the hose) to prevent flushing fertilizer over the edge of the pots. This fertilizer will usually last for approximately 2 weeks (or until the fertilizer particles start to appear translucent), at which time another application should be made.

A.7 Watering

A.7.1 How often should I water my plants?

To prevent plant stress, and thus to grow well-behaved waterhemp under herbicide treatment, plants should be watered at least daily in most cases. One notable exception to this

rule is when plants are in the seedling stage before the initial transplanting. At this stage the most important aspect of watering is just that the soil should always remain damp. During sunny days and especially in the summer this may mean watering each day, while at other times perhaps once every two days will suffice. Regardless of the amount of watering necessary, the plants should be observed at least once daily. After the initial transplanting—especially beginning several days afterward—the plants should be watered at least once per day every day. As a general rule, I water plants once per day when they are growing in 08-06 inserts, and I water them twice per day once they have been transplanted to pots.

A.7.2 How much water should I apply?

In the past four years I am not sure that I have ever observed a waterhemp plant suffering from too much watering. Therefore, my suggestion is, as I have discussed with my labmates in the past, that the plants should be flooded each time that they are watered. This is particularly true for plants growing in pots, while, realistically, seedlings should probably not be watered so heavily—as long as the soil is damp in the containers in which seeds were sown, the seedlings should be fine. When watering plants in pots, I generally spray water until it stands in the pots above the soil. This should not be a cause for concern due to the holes at the bottom of the pots—this excess water will soon drain, and the plants will not drown. This method of watering removes the guesswork involved in attempting to apply just the right amount of water to a plant to allow it to last until the next watering without wilting, and it allows the grower to think about other matters instead of worrying about whether the plants received enough water.

A.8 Lighting

I have never spent much time in experimenting with lighting for waterhemp growth, but I have observed that the plants begin flowering sooner in the winter when the days are shorter. I have also observed some inexplicable differences in plant responses to herbicides between runs of identically performed experiments, and differences in levels of sunlight have been suggested as a possible cause. During greenhouse work, particularly during the summer, it can become quite warm in the greenhouse, and turning off the lights can provide some much-needed relief. This should be an acceptable practice. However, it is recommended that the grower remember to turn the lights back on when leaving the greenhouse. This may seem like a simple enough prescription to follow, but it is surprising what one may forget when dealing with these plants in a greenhouse setting.

A.9 Herbicide Application

A.9.1 What should I do to prepare for herbicide application?

As a general guideline, plants should have reached the 10–15 cm growth stage at approximately 4 weeks after seeds were sown. This is the time (usually) at which herbicides should be applied to the plants. This can be a stressful time, particularly during the semester in trying to coordinate schedules with undergraduates, and therefore it is advisable to be prepared for this time before it arrives. Few things can so quickly make an otherwise good day turn bad as when one finds that the required herbicide or adjuvant has all been used or has been misplaced. I recommend locating all necessary supplies for herbicide application on the day before the planned application date. This allows for time to retrieve necessary materials from the south farm or the weed science laboratory before they are needed and can make herbicide application a

much smoother process when the time comes. I also recommend watering plants immediately upon arrival to the lab on the day that the plants are to be sprayed.

A.9.2 How much herbicide will I need to mix?

For details on the amount of herbicide that one needs to mix to apply a given treatment, see Appendix C. However, as a general rule, when plants have reached 10–15 cm in height, approximately 20 pots can be placed on the platform with little or no overlap of leaves from separate plants. The sprayer applies approximately 40 mL of spray solution per pass, and it is wise to mix some extra herbicide in case of unforeseen problems. I generally mix enough herbicide for at least one extra pass.

A.9.3 How do I mix herbicides?

Mixing herbicides often takes more time than expected, particularly for a novice. This can make things quite stressful when attempting to coordinate with the schedule of an undergraduate employee for help during spraying, which can easily lead to errors in calculations or mixing. Therefore, it is wise to perform the required mixing calculations the day before herbicide application is scheduled. Appendix C provides sample calculations for each of the herbicides that I used most frequently, along with "reduced mixing equations" in which all constants have been combined into a single number, leaving dose and mix volume as variables. Although it would be wise for the grower to practice some of these calculations the long way, reliance on the reduced mixing equations in an emergency will provide a safe and time-efficient method of calculation for the amount of herbicide to use for a given mix. In fact, after creating these reduced equations, I performed the calculations the long way only a couple more times to

check their accuracy, and then I relied almost solely on the reduced equations to calculate the proper mixes for the rest of my graduate career in the Tranel lab.

A.9.4 Mixing is finished. What should I take to the greenhouse?

One commonly forgotten, but necessary, supply is that of 40 mL vials. These are necessary, as these are essentially the spray tank for the sprayer—the herbicide applied to the plants by the nozzle comes directly from the 40 mL vials. I recommend taking at least 2 or perhaps 3 of these down to the greenhouse when spraying. Also, the grower should wear gloves during herbicide application and subsequent handling of the treated plants and pots, so gloves should also be taken to the greenhouse. It is advisable to take one or two extra pairs in case the original gloves become torn or must be removed for some reason. Of course, the other essential supply to take to the greenhouse for herbicide applications is the herbicide itself.

A.9.5 How should I spray my plants?

Assuming the grower has raised healthy, unstressed plants, it is now time to treat them with herbicide. See Appendix C for detailed information on operating the spray chamber.

A.9.6 When can I water my plants again?

After spraying, it is important to remember not to water the plants immediately, as this may reduce the effect of the herbicide. Therefore, I recommend watering all plants immediately upon arrival on the day that they are to be treated with herbicide. If the soil in the pots is fairly wet during spraying, I usually wait until morning to water the plants again. However, if the plants will need to be watered again on the same day of treatment, this should be done carefully

using a slow stream from the hose that can be applied directly to the soil in each pot without contacting the leaves.

A.9.7 When will I know if the herbicide treatment worked?

For some herbicides, such as lactofen or paraquat, some symptoms of injury will be visible within an hour or two after treatment. For other, slower acting herbicides such as glyphosate or imazamox, symptoms may not be visible until 2–3 days after treatment, although occasionally symptoms on a few plants may be seen after about one day.

A.10 Results

A.10.1 How long should I wait before collecting data?

The general rule is to wait until approximately 14 days after treatment before collecting data on the response of plants to a herbicide. In some cases this time may be shortened, but from my experience it never needs to be lengthened. In the case of application of fast-acting herbicides such as lactofen, it may be tempting to collect data in as little as a few days after treatment. However, this is unwise, as even the resistant plants often show high levels of injury after application of this herbicide. Sometimes what separates the susceptible from the resistant plants is the speed with which the resistant plants recover after treatment, but these plants must be given enough time to show this effect—otherwise they may be rated incorrectly as susceptible plants. For other herbicides, such as glyphosate, the injury symptoms take more time to appear. However, I have found that by waiting much more than 14 days after treatment to collect data, the main effect is that plants that would have (and should have) been rated as susceptible may begin to regrow and thus are rated as resistant. In a field setting, such plants probably would

have become shaded or otherwise outcompeted either by the crop or by other plants before the regrowth could have occurred and thus they would have died. Therefore, in most cases I recommend waiting at least 12 days, but no more than 16–18 days after treatment to collect data.

A.10.2 Are these plants resistant or susceptible?

I have found myself asking this same question multiple times over the course of the last four years. At times it can be difficult to tell for sure. However, confidently rating plants as resistant or susceptible passes from perhaps somewhat difficult to essentially impossible without the use of resistant and susceptible waterhemp populations as controls in each experiment. Hopefully, if the waterhemp plants have been grown stress-free until this point, then all of the susceptible control plants have been well controlled by the herbicide, making the job of rating plants much easier. In this case, essentially any plant that survived the herbicide treatment should be rated as resistant. A gray area may always be found, however, in which a plant almost died, but not quite. Whether such a plant is classified as resistant or susceptible in this case may depend upon the purpose of the experiment—I would lean toward the more conservative rating in any such case. For instance, if the experiment was being conducted to show that a population was resistant to a particular herbicide, I would probably call such barely-surviving plants susceptible. However, if an experiment is being conducted, for instance, to test the efficacy of a newly developed herbicide, I would probably take note of the survival of the plant and perform another experiment to get a better idea of whether the plant survived due to failure of the herbicide or due to other reasons.

In cases in which the susceptible control plants are not totally killed by the herbicide, the rating of plants becomes much more difficult, although in such cases it is still wise to attempt to

rate the plants, rather than wasting all of the work that went into conducting the experiment. In such cases, rather than collecting R or S ratings, it may be more wise to use dry weights or visual ratings to record the results of the experiment (see below).

A.10.3 What sort of data should I collect?

For most herbicide treatments, I assign a rating of resistant (R) or susceptible (S) to each plant at the designated time after treatment. This is often sufficient, and it is usually quite possible after treatment with herbicides such as imazamox, lactofen, or atrazine. In the case of glyphosate, however, it is much more difficult to assign an R or S rating. The R and S control plants help, but due to a broad range of phenotypes in response to glyphosate treatment, I have found that it is easier to record a response to glyphosate by assigning a visual rating to each plant between 0 (meaning no injury) and 10 (a crisp plant). To ensure consistency in visual rating assignments across an entire run, I have found that it is best first to physically group plants by injury symptoms (into 11 groups) and to then record the visual ratings for each plant. In addition, dry weight data may be useful (for glyphosate, as well as for other herbicide treatments), but whether this is necessary depends on the purpose of the experiment. Visual ratings may also be useful for results of treatment with herbicides other than glyphosate, again depending on the purpose of the experiment.

A.11 Flowering

A.11.1 When will my plants begin to flower?

After rating herbicide-treated plants, usually 4–6 more weeks will be required for flowering to begin. A notable exception to this loosely framed rule is WCS, which, in a few

cases, has even been observed to begin flowering before herbicide treatment. It is generally true that flowering among plants that were sown at the same time in a particular experiment can vary widely (the last plants to flower will likely begin flowering at least one month after the first plants began to flower). Therefore, when crossing is required, it is advisable to perform multiple identical experiments (four or more) staggered in time by about one week to ensure that an adequate supply of flowering plants are available for crossing when required.

A.11.2 How can I tell if a plant is male or female?

At the onset of flowering, identification of male or female plants is quite difficult for a novice. With practice, however, one can become quite proficient at determining the sex of a plant—perhaps with as high as a 95% success rate. It is difficult to describe the methodology in determining which plants are males and which are females, but the general idea is that male flower branches tend to have a larger diameter than do female flower branches. Also, the male flowers on such branches tend to be grouped into larger clusters with more space between the clusters (with the spaces between clusters increasing as the plant approaches the stage at which anthers become visible) than the female flowers, which tend to be located essentially continuously along the flower branch (although there is indeed some clustering that occurs). Another telltale sign that a plant is a female is the presence of stigmas, which are often visible upon careful inspection soon after flowering begins. When two plants have recently began flowering and are placed side by side, with one male and one female, the presence of the stigmas, although difficult to identify as such on a single plant, becomes readily apparent on the partially developed female flower when compared with the partially developed male flower. Especially for a novice, determining the sex of waterhemp plants before pollen production begins requires

close and frequent observation (and practice). As with most skills, practice makes (nearly) perfect, and the grower should not become discouraged by early failures.

A.11.3 How do I cross my male and female plants?

When plants are to be used for crossing, it is important that female plants are removed from undesired pollen sources before pollination occurs. Probably it is true that if a plant can be identified as a female, and if pollen is present in the same room, the female is capable of being, and perhaps already has been, pollinated. In such cases, if flowering has just begun, the new female flower can simply be plucked from the top of the plant. The plant should somehow be marked as a female (on the tag, or in some other simple way), and it should then be moved to a room away from the unwanted pollen.

Multiple female plants can be included in a cross with either a single male or even with multiple males depending on the purpose of the cross. For instance, if a cross is being performed to study the inheritance of a type of herbicide resistance, it is necessary that only one male be used in the cross. However, if a cross is being performed to increase seed stock of a particular line, in some cases it may be acceptable to include multiple males with the females to maximize seed production. When the types and numbers of plants to be used in a particular cross have been determined and their sexes identified, it is time to set up the cross.

Ideally, crosses should be performed in a growth chamber to minimize the potential for female plants to be pollinated by pollen from males other than the one selected for the particular cross. However, with the current state of the growth chambers in the basement of the greenhouse, the grower will quickly find that performing crosses in these chambers is not conducive to high seed production. This is mainly due to low light in the chambers. Many of the light sockets are

dysfunctional and plants grown in the chambers become etiolated and produce very few seeds and little pollen. Therefore, I recommend performing crosses in the greenhouse using pollination bags. Past tests I have performed have suggested that in the worst case scenario, pollen contamination among pollination bags may amount to approximately 200 seeds produced on an individual female, while in most cases this figure will be much smaller and essentially insignificant (Chapter 3).

Figure A.7 shows an example of a PVC pipe frame that I have used in the past to support pollination bags during crosses. The pollination bag should be placed over the frame, and one side of the bag should then be placed under the pipes to cover the bottom of the enclosure. Plants to be used in a cross can then be placed between the pipes making up the frame. It may be tempting to fill all of the available space in the enclosure with plants, but from past experience I can say that this is not advisable. Plants grown in close proximity during crossing experience one of two fates. In one case, a few of the plants may be outcompteted and will produce little if any seed. In the other case, the plants become thoroughly intertwined as they continue to grow while the crossing is in progress, making harvesting very difficult and time consuming. This tangling of plants during crosses has the added negative effect of leading to seed loss and potential contamination between seed lines when plants are untangled during harvesting. Therefore, it is recommended that the female plants be placed as far apart as possible within the enclosure and that the number of plants contained within an enclosure is limited to only those that are necessary—particularly in the case of genetics studies where seed contamination among females present within the same cross is undesirable (as opposed to a cross being performed for increasing seed supply, in which case the tangling of plants becomes less of a problem, as tangled branches may be cut together and placed in the same bag).

Once the plants have been placed in the enclosure, it should be closed by pulling the pollination bag completely over the frame, and tucking the edges of the bag opening under the frame to hold them down and keep the enclosure sealed. During watering, the grower should be careful to prevent pollen contamination among separate crosses. Viable seed should be produced within 14 days of pollination of female plants. Seed rain usually begins by 16 days after pollination. If rapid advancement of generations is the goal, then seeds may be harvested by 14 days after pollination. However, if higher seed production is of importance, then it may be advisable to allow the cross to occur for one or even two months, as the females continue to produce new flowers during the crossing.

One easily forgotten, but potentially important, step during crossing is for the grower to collect tissue samples from all parents. Even if no molecular markers currently exist for the type of resistance that the grower is studying, such markers may be discovered before the end of the grower's time as a graduate student, in which case having tissue samples of all parents may be beneficial. Therefore, I strongly recommend collecting these tissue samples and storing them at -80 C in case they may someday be needed. In fact, due to the potential importance of such samples, I recommend collecting two samples from each parent (stored in separate containers) so that if a problem occurs during DNA extraction from one of the samples, another sample will still be available for use. (This recommendation comes from direct experience with a similar situation, in which a bad batch of CTAB extraction buffer was used, resulting in the extraction of little or no DNA from several important parent plants.)

A.12 Communication

Communication is important in nearly every facet of life, and greenhouse work is no exception. Some of the most important topics of communication among labmates sharing greenhouse space should be on soil use, watering responsibilities, and coordination of the use of space in the greenhouse room. These topics are addressed below.

A.12.1 Where did all of our soil go?

Few problems can bring an experiment to a premature end like making the discovery that the soil stock has been depleted. From the time a soil order is placed, one must generally wait at least one week before the new soil is delivered. (Although in cases of an emergency, this interval may be considerably reduced if the grower has spent adequate time in building positive relationships with the greenhouse staff (see section below).) In the case where the grower is preparing to begin an experiment, this situation is not as critical as it may be in other cases. Simply waiting a week to plant the seeds will always solve the problem. However, for a case in which waterhemp plants are currently growing and are in need of transplanting, the situation is more serious. The plants will not wait to grow, and waiting one week to transplant plants that should be transplanted immediately will likely lead to those plants becoming stressed, which may negatively affect the outcome of the experiment in progress.

This is a problem that can easily be avoided by practicing good communication skills with one's labmates. The grower should be aware of other projects both planned and in progress by other members of the lab, and lab members should discuss soil needs well ahead of the time when the soil will actually be used. The soil use estimates of 400 4.5" pots per small soil cart seem to be fairly reliable during such planning meetings. (The same planning should also be

practiced for pot use, as a lack of pots may potentially lead to the same problems as does a lack of soil.)

A.12.2 I just got here—why are my plants wet?

Watering can often be a source of confusion with greenhouse work, particularly when it comes to watering another person's plants. Myself and several labmates have made it a practice to water everyone's plants when we water our own. This is usually acceptable, with a notable exception being immediately after a labmate has applied herbicide to his or her plants, in which case the plants should not be watered for at least a couple of hours. Therefore, again, communication can be important in these cases. The grower should have some idea of what experiments are being conducted by other members of the lab, and what the watering requirements are for such projects. Proper etiquette suggests that the grower should water the plants of labmates if they seem too dry. This can save headaches later, as the grower will not have to hesitate to ask other members of the lab to water his or her plants in times of need. And if for some reason the grower wishes for his or her plants to not be watered, he should tell this to other members of the lab to prevent any possible problems.

A.12.3 Where did all of these plants come from?

Of all the problems that a grower may experience due to sharing supplies and greenhouse space with labmates, this can be one of the most serious, as most experiments, once started, last for at least 6 weeks, with at least 3 of those weeks requiring plants to be in pots which tend to use a considerable amount of bench space in the greenhouse. For experiments to run smoothly (i.e. to ensure that enough space will be available for the grower to conduct an experiment), the grower

should make a habit of discussing greenhouse space needs with labmates. This should be done well in advance, as once seeds are planted it is impossible to postpone an experiment. This is particularly a problem for experiments being conducted with populations for which the seed stock is very limited. As a general rule, one can expect to fit between 500 and 800 pots on a bench (in room 1707), depending on how closely the pots are placed to one another. With this in mind, the grower should keep track of both experiments being conducted—and of experiments being planned—by other members of the lab. Doing so will allow the grower to be more productive, and will help to keep tensions to a minimum.

A.12.4 Relationship with greenhouse staff

Building a good rapport with the greenhouse staff is essential to smooth work in greenhouse. I have experienced several benefits from such a relationship, including quickly receiving soil in the case of an emergency, flexibility in the time of application of pest control chemicals by members of the greenhouse staff, emergency watering of my plants being performed by members of the greenhouse staff, and in general, more leniency with the rules, which can often help the grower accomplish necessary tasks more easily or more quickly. Building such a relationship may also have some benefits in cases when it is necessary that extra temporary greenhouse space be located for the grower. Therefore, it is recommended that the grower talk with greenhouse staff, and "put up" with their occasional scolding—particularly early on in the grower's career as a graduate student. These scoldings can be kept to a minimum if the grower cleans up after himself in the greenhouse hallway as well as in the greenhouse room itself. In fact, the cause of many of the scoldings that I have received in the past have been due to soil on the greenhouse room floor or due to storing of extra supplies within the

greenhouse room itself. It is recommended that the grower transport all leftover supplies back to the storeroom after use. This simple step can greatly improve relations with greenhouse staff and ensure that greenhouse work will continue to go smoothly for the grower.

A.13 Troubleshooting

When growing waterhemp for greenhouse studies, one soon learns that Murphy's Law is not blocked out by greenhouse glass. It applies within the greenhouse as well as without. This section is meant to address the potential causes of some common problems associated with waterhemp growth in the greenhouse.

A.13.1 Why is the water just sitting on top of the soil?

If soil is not used within 2–3 weeks after ordering, it tends to become dry—especially in the summer when temperatures in the greenhouse (where the soil is stored) can become quite high on a sunny day. If this dry soil condition is not remedied, it can lead to substantial delays (on the order of hours at least, and up to one whole day) in any procedure requiring the use of soil, as for some reason the soil tends to actually repel water when it has become too dry. However, with a little foresight this problem can be prevented. One measure that can be taken to prevent such a problem is to only order as much soil as is needed for a certain project. However, small projects, either unforeseen or temporarily forgotten, will invariably arise, which require the use of soil in small quantities. Therefore, it is wise to always keep some soil on hand, despite the fact that this soil will likely become too dry for immediate use by the time it is needed.

By periodically monitoring the soil conditions this problem can be prevented. If the soil appears or feels dry, the grower should water the soil (assuming it has been transferred from the

cart/wagon in which it was delivered into a white plastic soil tub) with one of the numerous hoses that can be found in the main hallway of the greenhouse. In preparation for watering, the soil in the tub should be leveled to prevent pooling of water into certain pockets. Then the soil can be covered with water. The amount applied will obviously depend on the volume of the soil in the tub. It will be left as an exercise for the grower to determine the proper amount to add, but it is wise to err on the side of too little water rather than too much. The method that I have found to work best is to cover the soil with water, replace the cover of the tub, and let the soil remain undisturbed for a day. Then on the next day, the soil should be thoroughly mixed, with special attention paid to the soil at the bottom of the tub, which will likely be saturated by this time, as well as the soil in the middle of the tub which may still be quite dry, as the water tends to only sink to the bottom in certain regions of the tub (presumably either along the edges or in the few areas where the soil near the surface was not quite as dry as the surrounding soil). This mixing is most easily performed if there is another empty soil tub available, in which case the soil can be transferred from one tub to another, with mixing occurring during the process, although if no other tub is available, the soil can still be mixed with minor added difficulty. After mixing the soil, assuming that enough water was added, the soil should be ready for use, meaning that it will readily absorb water applied to its surface. Soil which is ready to be used should feel moist or damp to the touch. Soggy soil will also serve the purpose, but such soil can easily become compacted, which may have some negative effects on plant growth.

A.13.2 Why have my seeds not germinated?

This problem is likely due to insufficient stratification time. By waiting for longer than usual after sowing, some seeds should germinate. However, in some cases this problem could

also be due to fermentation of stratified seeds, caused by insufficient sterilization during stratification. In this case (obvious by the smell of the seeds in the tube), little or no germination should ever be expected. A lack of germination may also be caused by not keeping the soil in which the seeds were sown moist enough.

A.13.3 Why did my seedlings die?

There are many potential causes for this problem. One of the most likely is either that the seedlings did not receive enough water, or that they were watered too harshly. If the seedlings died after transplanting, the cause is likely that the soil into which they were transplanted was not thoroughly wetted first, or that the delicate seedlings were not handled with enough care during the transplant. Greenhouse pests such as fungus or insects could also potentially cause such problems.

A.13.4 Why will my seedlings not stand up straight?

This is a common symptom of not burying the seedlings deep enough during the initial transplant. I recommend leaving only approximately the top 1 cm of transplanted seedlings exposed above the soil surface. This helps to stabilize the seedlings, preventing them from falling over during watering. If the seedlings were initially placed at the recommended depth and they are still buried, this is likely due to too much force during watering. Such seedlings are still quite fragile for several days after the initial transplanting, and they should be watered gently with a watering can.

A.13.5 Why are there brown spots on the leaves?

This is a common symptom of injury due to thrips. This usually is not a major cause for concern, but the greenhouse staff does offer pest control, and asking them to take care of the thrip problem may be desirable in such cases.

A.13.6 Why are my plants all different heights?

As noted earlier, this is a common problem with growing waterhemp plants. Although selection for uniformity during each of the transplanting stages helps, some variability in plant height is still to be expected up until the time of treatment. The two-transplant method is an attempt to remedy this problem, and the grower will easily discover that, by eliminating the first transplant and placing seedlings directly into pots, a wide range of plant heights will be obvious within one week after transplanting. This requires many more pots to be filled than would otherwise be necessary to allow for selection of an adequate number of uniform plants for herbicide treatment, and thus, although more time is required for performing two transplants, this method is still highly recommended.

A.13.7 Why did the susceptible controls survive?

Of all the questions that might arise during experimentation on waterhemp, this is perhaps the most dreaded question I have been asked and that I have had to ask myself during the last four years. In some cases, the lack of control of the susceptible plants may be due to plant stress. The grower should ensure that large enough pots are being used, that plants are watered frequently, that plants are adequately fertilized, and that other potential stressors are kept to a minimum. However, sometimes, even after receiving the best care possible, the susceptible

control plants may inexplicably survive treatment with an herbicide. I have found this to be particularly true with glyphosate. In one run of an experiment ACR (the susceptible control) may be effectively controlled with a ½x rate of glyphosate, while in a second run with just a one week delay from the first, ACR may require a 2x rate to be effectively controlled. This effect was quite obvious in the results and discussion section of Chapter 5. In these cases, it is difficult to even speculate on the causes. The only potential leads that I have come up with are those of uncontrollable environmental factors such as the amount of sunlight and relative humidity levels. It would be interesting to keep track of such conditions to test whether some correlation exists between these and a plant's response to glyphosate. However, such problems are a reality of greenhouse work that the grower must deal with, and even after less than ideal responses displayed by susceptible control plants, the grower should attempt glean at least some valuable information from such experiments to which he or she has presumably devoted much time and effort.

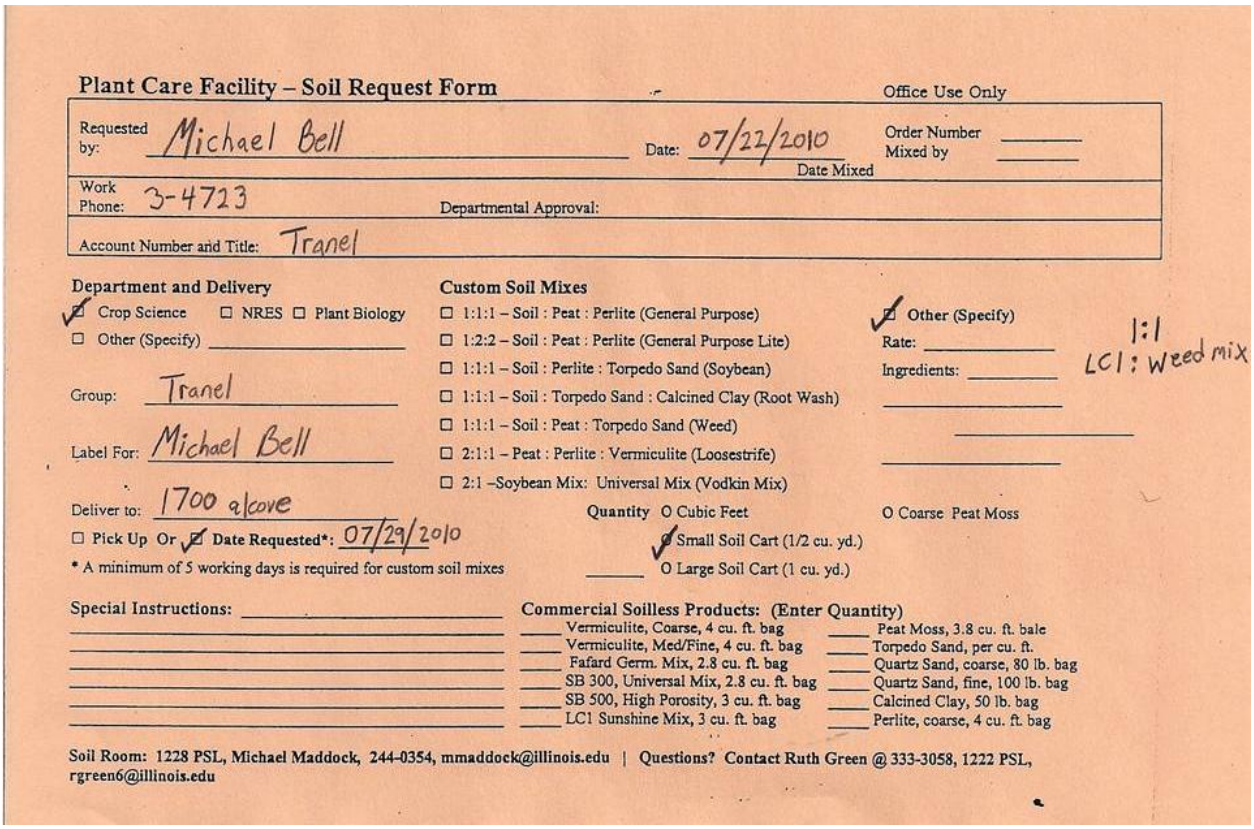
A.14 Some Final Words of Advice

As can be inferred from the preceding paragraphs, a student growing waterhemp for weed science research should "expect the unexpected". Many aspects of the research can be quite enjoyable at times—one aspect that I never grew tired of was watching the relatively rapid growth of these weeds from miniscule seeds into plants as much as 1 m tall! However, weed science research, particularly on waterhemp, is not as easy as simply sowing seeds, waiting, and then spraying plants with herbicide and observing which plants are resistant and which are susceptible. In order for experiments to be successful, the grower must be vigilant in many aspects of waterhemp development, ensuring that the plants are raised to experience as little

stress as possible. Ultimately, the most important thing that a grower can do to ensure that his or her waterhemp is grown properly is to carefully plan ahead. With a little foresight, most potential problems can be avoided, greatly improving the student's chances of growing healthy and well-behaved waterhemp plants.

A.15 Figures

Figure A.1 Barnstead EASYpure LF water purifier. This device should be used for water used during stratification. The machine is turned on by pushing the light green button on the far right labeled “START”. After turning the machine on, it should be allowed to run for approximately 30 seconds until the display (red numbers at top) reads at least 17.0 MΩ-cm. To start the flow of water, pull down the light green lever (pictured at left center). To stop the flow, the lever is pushed back up to the starting position. To turn off the machine, push the blue button on the left labeled “STOP”.



Plant Care Facility – Soil Request Form

Office Use Only

Requested by: Michael Bell Date: 07/22/2010 (Date Mixed) Order Number ____ Mixed by ____

Work Phone: 3-4723 Departmental Approval:

Account Number and Title: Tranel

Department and Delivery

☑ Crop Science ☐ NRES ☐ Plant Biology

☐ Other (Specify) ____

Group: Tranel

Label For: Michael Bell

Deliver to: 1700 alcove

☐ Pick Up Or ☑ Date Requested*: 07/29/2010

* A minimum of 5 working days is required for custom soil mixes

Custom Soil Mixes

☐ 1:1:1 – Soil : Peat : Perlite (General Purpose)

☐ 1:2:2 – Soil : Peat : Perlite (General Purpose Lite)

☐ 1:1:1 – Soil : Perlite : Torpedo Sand (Soybean)

☐ 1:1:1 – Soil : Torpedo Sand : Calcined Clay (Root Wash)

☐ 1:1:1 – Soil : Peat : Torpedo Sand (Weed)

☐ 2:1:1 – Peat : Perlite : Vermiculite (Loosestrife)

☐ 2:1 –Soybean Mix: Universal Mix (Vodkin Mix)

☑ Other (Specify)

Rate: 1:1

Ingredients: LC1 : Weed mix

Quantity O Cubic Feet O Coarse Peat Moss

☑ Small Soil Cart (1/2 cu. yd.)

____ O Large Soil Cart (1 cu. yd.)

Special Instructions: ____

Commercial Soilless Products: (Enter Quantity)

____ Vermiculite, Coarse, 4 cu. ft. bag

____ Vermiculite, Med/Fine, 4 cu. ft. bag

____ Fafard Germ. Mix, 2.8 cu. ft. bag

____ SB 300, Universal Mix, 2.8 cu. ft. bag

____ SB 500, High Porosity, 3 cu. ft. bag

____ LC1 Sunshine Mix, 3 cu. ft. bag

____ Peat Moss, 3.8 cu. ft. bale

____ Torpedo Sand, per cu. ft.

____ Quartz Sand, coarse, 80 lb. bag

____ Quartz Sand, fine, 100 lb. bag

____ Calcined Clay, 50 lb. bag

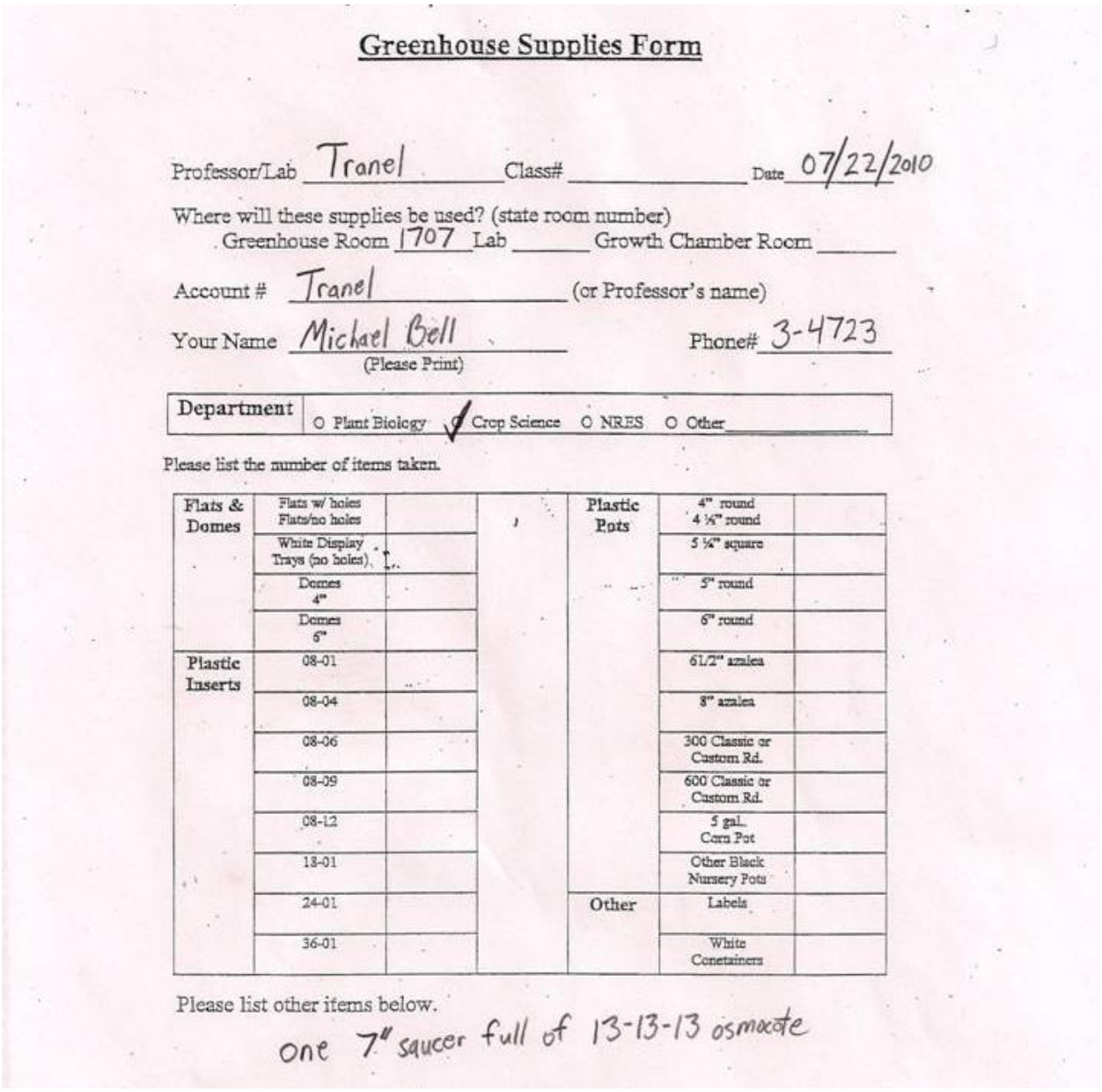
____ Perlite, coarse, 4 cu. ft. bag

Soil Room: 1228 PSL, Michael Maddock, 244-0354, mmaddock@illinois.edu | Questions? Contact Ruth Green @ 333-3058, 1222 PSL, rgreen6@illinois.edu

Figure A.2 The form used for ordering soil. The form should be filled out as shown to order the usual soil. The quantity can either be a small soil cart (as selected above) or a large soil cart, which is enough to fill two white soil tubs, or approximately 800 4.5" square pots.

Figure A.3 Small soil cart. This cart holds enough soil to fill approximately 400 4.5” square 700 mL pots, or enough to fill approximately 800 4” standard (green, round) pots.

Figure A.4 Plastic soil tubs. Soil should be transferred from soil carts to tubs soon after delivery.



Greenhouse Supplies Form

Professor/Lab Tranel Class# _____ Date 07/22/2010

Where will these supplies be used? (state room number)
Greenhouse Room 1707 Lab _____ Growth Chamber Room _____

Account # Tranel (or Professor's name)

Your Name Michael Bell (Please Print) Phone# 3-4723

Department	○ Plant Biology	✓ Crop Science	○ NRES	○ Other _____

Please list the number of items taken.

Flats & Domes	Flats w/ holes Flats/no holes			Plastic Pots	4" round 4 ½" round	
	White Display Trays (no holes)				5 ¼" square	
	Domes 4"				5" round	
	Domes 6"				6" round	
Plastic Inserts	08-01				6 1/2" azalea	
	08-04				8" azalea	
	08-06				300 Classic or Custom Rd.	
	08-09				600 Classic or Custom Rd.	
	08-12				5 gal. Corn Pot	
	13-01				Other Black Nursery Pots	
	24-01			Other	Labels	
	36-01				White Conetainers	

Please list other items below.

one 7" saucer full of 13-13-13 osmocote

Figure A.5 The form required to be filled out for use of containers and fertilizer. The 08-01 plastic inserts are used for sowing seeds. The 08-06 inserts are used for the initial transplantation. Generally flats w/ holes (with holes) are used for both of these steps. The procedure for reporting the use of fertilizer is depicted.

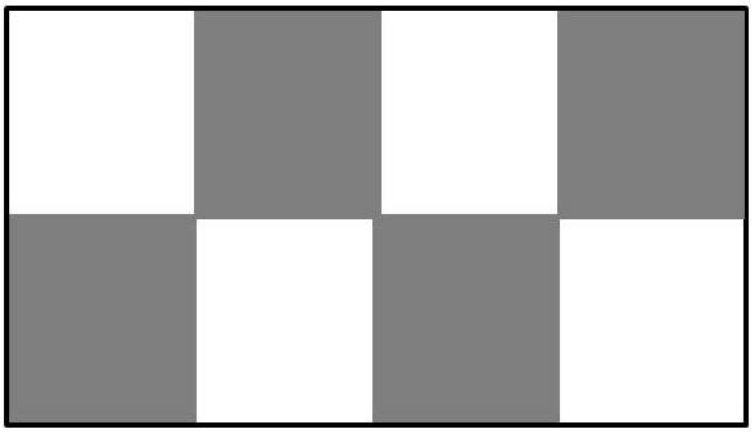


Figure A.6 Flat filled with 08-01 inserts, with inserts distributed in a checkerboard fashion to prevent contamination among seed lines during watering. Shaded squares represent soil-filled, seed-containing inserts.

Figure A.7 Example of a frame made of ¾” PVC pipe used to support a pollination bag during crossing of waterhemp plants in the greenhouse.

APPENDIX B

KEY TO SEED LINES

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
MO1	Original Field Population		F. MO
MO1	From greenhouse cross	F. MO	MO1
MO2	From greenhouse cross		MO2
Bulk	Random mating WCS (seed bulk)	WCS	WCS1
Bulk	Random mating WCS (seed bulk)	WCS	WCS2
Bulk	Random mating WCS (seed bulk)	WCS	WCS3
Bulk	Random mating WCS (seed bulk)	WCS	WCS4
Bulk	Random mating WCS (seed bulk)	WCS	WCS5
Bulk	Random mating ACR (seed bulk)	ACR	ACR1
Bulk	Random mating ACR (seed bulk)	ACR	ACR2
Bulk	Random mating ACR (seed bulk)	ACR	ACR3
Bulk	Random mating ACR (seed bulk)	ACR	ACR4
Bulk	Random mating ACR (seed bulk)	ACR	ACR5
Bulk	Random mating ACR (seed bulk)	ACR	ACR6
Bulk	Selfing SPS for seed-bulking	SPS #1	MBX87
Bulk	Selfing SPS for seed bulking	SPS #2	MBX88
TEST	ACR x ACR (Round 1)	ACR x ACR A	AA-A1
TEST	ACR x ACR (Round 1)	ACR x ACR A	AA-A2
TEST	ACR x ACR (Round 1)	ACR x ACR A	AA-A3
F_1	MO1 x ACR (Round 1)	MO1 x ACR A	1A-A1
F_1	MO1 x ACR (Round 1)	MO1 x ACR A	1A-A2
F_1	MO1 x ACR (Round 1)	MO1 x ACR A	1A-A3
F_1	MO1 x ACR (Round 1)	MO1 x ACR A	1A-A4
F_1	ACR x MO1 (Round 1)	ACR x MO1 A	A1-A1
F_1	ACR x MO1 (Round 1)	ACR x MO1 A	A1-A3

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
R x R	MO1 x MO2 (Round 1)	MO1 x MO2 A	12-A1
R x R	MO1 x MO2 (Round 1)	MO1 x MO2 A	12-A2
R x R	MO1 x MO2 (Round 1)	MO1 x MO2 A	12-A3
R x R	MO2 x MO2 (Round 1)	MO2 x MO2 A	22-A1
R x R	MO2 x MO2 (Round 1)	MO2 x MO2 A	22-A2
R x R	MO2 x MO2 (Round 1)	MO2 x MO2 A	22-A3
R x R	MO2 x MO2 (Round 1)	MO2 x MO2 A	22-A4
F_1	ACR x MO2 (Round 1)	ACR x MO2 A	A2-A1
F_1	ACR x MO2 (Round 1)	ACR x MO2 A	A2-A2
F_1	ACR x MO2 (Round 1)	ACR x MO2 A	A2-A3
F_1	ACR x MO2 (Round 1)	ACR x MO2 A	A2-A4
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 A	21-A1
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 A	21-A2
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 A	21-A3
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 A	21-A4
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 A	21-A5
F_1	MO2 x ACR (Round 1)	MO2 x ACR A	2A-A1
F_1	MO2 x ACR (Round 1)	MO2 x ACR A	2A-A2
F_1	MO2 x ACR (Round 1)	MO2 x ACR A	2A-A3
F_1	MO2 x ACR (Round 1)	MO2 x ACR A	2A-A4
F_1	MO2 x ACR (Round 1)	MO2 x ACR A	2A-A5
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 A	11-A2
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 A	11-A3
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 A	11-A4
TEST	ACR x ACR (Round 1)	ACR x ACR B	AA-B2
F_1	MO1 x ACR (Round 1)	MO1 x ACR B	1A-BR5C
F_1	MO1 x ACR (Round 1)	MO1 x ACR B	1A-BR6C
F_1	MO1 x ACR (Round 1)	MO1 x ACR B	1A-BR7C

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
F_1	MO2 x ACR (Round 1)	MO2 x ACR B	2A-BR5C
F_1	MO2 x ACR (Round 1)	MO2 x ACR B	2A-BR8C
F_1	MO2 x ACR (Round 1)	MO2 x ACR B	2A-BR9C
F_1	MO2 x ACR (Round 1)	MO2 x ACR B	2A-BR10C
F_1	MO2 x ACR (Round 1)	MO2 x ACR B	2A-BR13C
F_1	ACR x MO1 (Round 1)	ACR x MO1 B	A1-B1
F_1	ACR x MO1 (Round 1)	ACR x MO1 B	A1-B2
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR1C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR5C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR6C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR7C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR8C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR12C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR14C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 B	11-BR15C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 B	21-BR5C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 B	21-BR8C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 B	21-BR9C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 B	21-BR10C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 B	21-BR13C
F_1	ACR x MO1 (Round 1)	ACR x MO1 C	A1-C1
F_1	ACR x MO1 (Round 1)	ACR x MO1 C	A1-C2
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR1C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR5C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR6C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR7C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR8C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR12C

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR14C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 C	11-CR15C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 C	21-CR5C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 C	21-CR8C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 C	21-CR9C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 C	21-CR10C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 C	21-CR13C
F_1	ACR x MO1 (Round 1)	ACR x MO1 D	A1-D1
F_1	ACR x MO1 (Round 1)	ACR x MO1 D	A1-D2
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 D	11-DR6C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 D	11-DR7C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 D	11-DR8C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 D	11-DR12C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 D	11-DR14C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 D	11-DR15C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 D	21-DR5C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 D	21-DR8C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 D	21-DR9C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 D	21-DR10C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 D	21-DR13C
F_1	ACR x MO1 (Round 1)	ACR x MO1 E	A1-E1
F_1	ACR x MO1 (Round 1)	ACR x MO1 E	A1-E2
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER1C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER5C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER6C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER7C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER8C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER12C

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER14C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 E	11-ER15C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 E	21-ER5C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 E	21-ER8C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 E	21-ER9C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 E	21-ER10C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 E	21-ER13C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR1C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR5C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR6C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR7C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR8C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR12C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR14C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 F	11-FR15C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 F	21-FR5C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 F	21-FR8C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 F	21-FR9C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 F	21-FR10C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 F	21-FR13C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 G	11-GR1C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 G	11-GR5C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 G	11-GR6C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 G	11-GR8C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 G	11-GR14C
R x R	MO1 x MO1 (Round 1)	MO1 x MO1 G	11-GR15C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 G	21-GR5C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 G	21-GR8C

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 G	21-GR9C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 G	21-GR10C
R x R	MO2 x MO1 (Round 1)	MO2 x MO1 G	21-GR13C
F_1	ACR x C4 (Line A-222)	ACR x $C4_{A-222}$ A	$A4_A$-A1
F_1	ACR x C4 (Line A-222)	ACR x $C4_{A-222}$ A	$A4_A$-A2
R x R	C4 x C4 (Line A-222)^2	$C4_{A-222}$ x $C4_{A-222}$ A	4_A4_A-A1
F_1	ACR x C4 (Line A-432)	ACR x $C4_{A-432}$ A	$A4_B$-A1
F_1	ACR x C4 (Line A-432)	ACR x $C4_{A-432}$ A	$A4_B$-A2
R x R	C4 x C4 (Line A-432)^2	$C4_{A-432}$ x $C4_{A-432}$ A	4_B4_B-A1
R x R	C4 x C4 (Line A-432)^2	$C4_{A-432}$ x $C4_{A-432}$ A	4_B4_B-A2
F_1	ACR x C4 (Line A-432)	ACR x $C4_{A-432}$ B	$A4_B$-B1
F_1	ACR x C4 (Line A-432)	ACR x $C4_{A-432}$ B	$A4_B$-B2
R x R	C4 x C4 (Line A-432)^2	$C4_{A-432}$ x $C4_{A-432}$ B	4_B4_B-B1
R x R	C4 x C4 (Line A-432)^2	$C4_{A-432}$ x $C4_{A-432}$ B	4_B4_B-B2
TEST	ACR x ACR	ACR x ACR C	AA-C1
TEST	ACR x ACR	ACR x ACR C	AA-C2
F_1	C4 (Line A-432) x ACR	$C4_{A-432}$ x ACR C	4_BA-C1
F_1	C4 (Line A-432) x ACR	$C4_{A-432}$ x ACR C	4_BA-C2
TEST	ACR x ACR	ACR x ACR D	AA-D1
F_1	C4 (Line A-432) x ACR	$C4_{A-432}$ x ACR D	4_BA-D1
R x R	C4 x C4	$C4_{A-432}$ x $C4_{A-222}$ B	4_A4_A-B1
R x R	C4 x C4	$C4_{A-432}$ x $C4_{A-222}$ B	4_A4_A-B2
F_1	ACR x FCG (IL)	ACR x IL #1	MBX13
F_1	ACR x IL	ACR x IL #1	MBX32
F_1	ACR x IL	ACR x IL #2	MBX34
F_1	(MO1 x MO1) x ACR	11-A2 x ACR #246	MBX36
F_1	ACR x IL	ACR x IL #1	MBX39
F_1	ACR x (MO1 x MO1)	ACR x 11-A3 #164	MBX41

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
F_1	ACR x MO1 (Round 2)	ACR x MO1 #40	MBX45
F_1	MO1 x ACR (Round 2)	MO1 95B x ACR #2	MBX46
F_1	MO1 x ACR (Round 2)	MO1 21C x ACR #2	MBX47
F_1	MO1 x ACR (Round 2)	MO1 20B x ACR #2	MBX48
F_1	MO1 x ACR (Round 2)	MO1 42A x ACR #1	MBX50
F_1	MO1 x ACR (Round 2)	MO1 43A x ACR #1	MBX51
F_1	MO1 x ACR (Round 2)	MO1 77A x ACR #1	MBX52
F_1	MO1 x ACR (Round 2)	MO1 20A x ACR #1	MBX54
F_1	MO1 x ACR (Round 2)	MO1 95A x ACR #1	MBX55
F_1	MO1 x ACR (Round 2)	MO1 21B x ACR #1	MBX56
F_1	MO1 x ACR (Round 2)	MO1 42B x ACR #1	MBX59
F_1	MO1 x ACR (Round 2)	MO1 19C x ACR #1	MBX60
F_1	MO1 x ACR (Round 2)	MO1 119A x ACR #1	MBX61
F_1	MO1 x ACR (Round 2)	MO1 119B x ACR #2	MBX63
F_1	MO1 x ACR (Round 2)	MO1 43C x ACR #2	MBX65
F_1	MO1 x ACR (Round 2)	MO1 19B x ACR #2	MBX66
F_1	ACR x MO1 (Round 2)	ACR x MO1 #40	MBX68
F_1	MO1 x ACR (Round 2)	MO1 77B x ACR #2	MBX69
F_1	ACR x MO1 (Round 2)	ACR x MO1 #60	MBX82
F_1	ACR x MO1 (Round 2)	ACR x MO1 #60	MBX83
F_1	ACR x MO1 (Round 2)	ACR x MO1 #99	MBX92
F_1	ACR x MO1 (Round 2)	ACR x MO1 #99	MBX93
F_1	ACR x MO1 (Round 2)	ACR x MO1 #17	MBX135
F_1	ACR x MO1 (Round 2)	ACR x MO1 #17	MBX138
F_1	MO1 x ACR (Round 2)	MO1 19F x ACR #3	MBX148
F_1	MO1 x ACR (Round 2)	MO1 43H x ACR #3	MBX149
F_1	MO1 x ACR (Round 2)	MO1 20H x ACR #3	MBX151
F_1	MO1 x ACR (Round 2)	MO1 21H x ACR #3	MBX152

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
F_1	MO1 x ACR (Round 2)	MO1 42E x ACR #3	MBX153
F_1	MO1 x ACR (Round 2)	MO1 119F x ACR #3	MBX154
F_1	MO1 x ACR (Round 2)	MO1 77H x ACR #3	MBX155
F_1	MO1 x ACR (Round 2)	MO1 95G x ACR #3	MBX156
F_2	F_1 x F_1 (Round 1)	A1-A1 x A1-A1 #153	MBX2
F_2	F_1 x F_1 (Round 1)	A1-A1 x A1-A1 #153	MBX5
F_2	F_1 x F_1 (Round 1)	A1-A1 x A1-A1 #62	MBX16
F_2	F_1 x F_1 (Round 1)	A1-A1 x A1-A1 #62	MBX18
F_2	F_1 x F_1 (Round 1)	A1-A1 x A1-A1 #153	MBX20
F_2	F_1 x F_1 (Round 2) (MBX52)[2]	F_1 #101 x F_1 #5	MBX157
F_2	F_1 x F_1 (Round 2) (MBX52)[2]	F_1 #135 x F_1 #5	MBX158
F_2	F_1 x F_1 (Round 2) (MBX63)[2]	F_1 #52 x F_1 #154	MBX159
F_2	F_1 x F_1 (Round 2) (MBX52)[2]	F_1 #205 x F_1 #5	MBX160
F_2	F_1 x F_1 (Round 2) (MBX63)[2]	F_1 #10 x F_1 #154	MBX161
F_2	F_1 x F_1 (Round 2) (MBX52)[2]	F_1 #134 x F_1 #5	MBX162
F_2	F_1 x F_1 (Round 2) (MBX63)[2]	F_1 #19 x F_1 #154	MBX163
F_2	F_1 x F_1 (Round 2) (MBX52)[2]	F_1 #6 x F_1 #5	MBX164
F_2	F_1 x F_1 (Round 2) (MBX61)[2]	F_1 #8 x F_1 #237	MBX165
F_2	F_1 x F_1 (Round 2) (MBX63)[2]	F_1 #51 x F_1 #154	MBX166
F_2	F_1 x F_1 (Round 2) (MBX63)[2]	F_1 #29 x F_1 #154	MBX167
BC_R	(MO1 x MO1) x Round 1 F_1	11-A1 x A1-A1 #153	MBX1
BC_R	(MO1 x MO1) x Round 1 F_1	11-A1 x A1-A1 #153	MBX3
BC_R	(MO1 x MO1) x Round 1 F_1	11-A1 x A1-A1 #62	MBX15
BC_R	(MO1 x MO1) x Round 1 F_1	11-A2 x A1-A1 #62	MBX21
BC_S	ACR x Round 1 F_1	ACR x A1-A1 #153	MBX4
BC_S	Round 1 F_1 x ACR	A1-A1 x ACR #246	MBX6
BC_S	Round 1 F_1 x ACR	A1-A1 x ACR #246	MBX7
BC_S	Round 1 F_1 x ACR	A1-A1 x ACR #246	MBX14

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
BC_S	ACR x Round 1 F_1	ACR x A1-A1 #62	MBX17
BC_S	(ACR x ACR) x Round 1 F_1	AA-A3 x A1-A1 #62	MBX19
R x R	MO1 x IL	MO1 x IL #1	MBX27
R x R	IL x IL	IL x IL #1	MBX28
R x R	IL x IL	IL x IL #1	MBX29
R x R	MO1 x IL	MO1 x IL #1	MBX30
R x R	IL x IL	IL x IL #1	MBX31
R x R	MO1 x IL	MO1 x IL #2	MBX33
R x R	MO1 x IL	MO1 x IL #2	MBX35
R x R	(MO1 x MO1) x (MO1 x MO1)	11-A2 x 11-A3 #164	MBX37
R x R	(MO1 x MO1) x IL	11-A2 x IL #2	MBX38
R x R	(MO1 x MO1) x (MO1 x MO1)	11-A3 x 11-A3 #164	MBX40
R x R	(MO1 x MO1) x (MO1 x MO1)	11-A3 x 11-A3 #164	MBX42
R x R	(MO1 x MO1) x (MO1 x MO1)	11-A2 x 11-A3 #164	MBX43
R x R	(MO1 x MO1) x IL	11-A1 x IL #1	MBX44
R x R	MO1 x MO1 (Round 2)	MO1 95D x MO1 #40	MBX57
R x R	MO1 x MO1 (Round 2)	MO1 42C x MO1 #40	MBX62
R x R	MO1 x MO1 (Round 2)	MO1 19D x MO1 #40	MBX64
R x R	MO1 x MO1 (Round 2)	MO1 47D x MO1 #40	MBX67
R x R	MO1 x MO1 (Round 2)	MO1 20C x MO1 #40	MBX70
R x R	MO1 x MO1 (Round 2)	MO1 20D x MO1 #40	MBX71
R x R	MO1 x MO1 (Round 2)	MO1 119C x MO1 #40	MBX74
R x R	MO1 x MO1 (Round 2)	MO1 77C x MO1 #40	MBX76
R x R	MO1 x MO1 (Round 2)	MO1 21E x MO1 #60	MBX77
R x R	MO1 x MO1 (Round 2)	MO1 95E x MO1 #60	MBX78
R x R	MO1 x MO1 (Round 2)	MO1 20D x MO1 #60	MBX79
R x R	MO1 x MO1 (Round 2)	MO1 42D x MO1 #60	MBX80
R x R	MO1 x MO1 (Round 2)	MO1 19G x MO1 #60	MBX81

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
R x R	MO1 x MO1 (Round 2)	MO1 43E x MO1 #60	MBX84
R x R	MO1 x MO1 (Round 2)	MO1 77D x MO1 #60	MBX85
R x R	MO1 x MO1 (Round 2)	MO1 119E x MO1 #60	MBX86
R x R	MO1 x MO1 (Round 2)	MO1 42G x MO1 #99	MBX89
R x R	MO1 x MO1 (Round 2)	MO1 77F x MO1 #99	MBX90
R x R	MO1 x MO1 (Round 2)	MO1 21G x MO1 #99	MBX91
R x R	MO1 x MO1 (Round 2)	MO1 119G x MO1 #99	MBX94
R x R	MO1 x MO1 (Round 2)	MO1 19H x MO1 #99	MBX134
R x R	MO1 x MO1 (Round 2)	MO1 21F x MO1 #17	MBX136
R x R	MO1 x MO1 (Round 2)	MO1 77E x MO1 #17	MBX137
R x R	MO1 x MO1 (Round 2)	MO1 119H x MO1 #17	MBX139
R x R	MO1 x MO1 (Round 2)	MO1 47H x MO1 #17	MBX140
R x R	MO1 x MO1 (Round 2)	MO1 43F x MO1 #17	MBX141
R x R	MO1 x MO1 (Round 2)	MO1 20E x MO1 #17	MBX142
R x R	MO1 x MO1 (Round 2)	MO1 95F x MO1 #17	MBX143
R x R	MO1 x MO1 (Round 2)	MO1 20G x MO1 #99	MBX144
R x R	MO1 x MO1 (Round 2)	MO1 43G x MO1 #99	MBX145
R x R	MO1 x MO1 (Round 2)	MO1 95H x MO1 #99	MBX146
R x R	MO1 x MO1 (Round 2)	MO1 19E x MO1 #17	MBX147
TEST	Isolated ACR (Round 2)	ACR x foreign pollen	MBX53
TEST	ACR x ACR (Round 2)	ACR x ACR #1	MBX58
TEST	ACR x ACR (Round 2)	ACR x ACR #2	MBX72
TEST	Isolated ACR (Round 2)	ACR x foreign pollen	MBX73
TEST	ACR x ACR (Round 2)	ACR x ACR #3	MBX150
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #2	MBX8
Hybrid	(MO1 x MO1) x SPS (Round 1)	11-A2 x SPS #2	MBX9
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #2	MBX10
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #2	MBX11

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #2	MBX12
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #1	MBX22
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #1	MBX23
Hybrid	(MO1 x MO1) x SPS (Round 1)	11-A2 x SPS #1	MBX24
Hybrid	MO1 x SPS (Round 1)	MO1 x SPS #1	MBX25
Hybrid	(ACR x ACR) x SPS (Round 1)	AA-A1 x SPS #1	MBX26
Hybrid	MO1 x SPS (Round 2)	MO1 117 x SPS #1+2	MBX49
Hybrid	MO1 x SPS (Round 2)	MO1 77 x SPS #1+2	MBX75
4-Way	Random mating 4-way R MBX5	MBX5	MBX95
4-Way	Random mating 4-way R MBX5	MBX5	MBX96
4-Way	Random mating 4-way R MBX5	MBX5	MBX97
4-Way	Random mating 4-way R MBX5	MBX5	MBX98
4-Way	Random mating 4-way R MBX5	MBX5	MBX99
4-Way	Random mating 4-way R MBX5	MBX5	MBX100
4-Way	Random mating 4-way R MBX5	MBX5	MBX101
4-Way	Random mating 4-way R MBX5	MBX5	MBX102
4-Way	Random mating 4-way R MBX5	MBX5	MBX103
4-Way	Random mating 4-way R MBX5	MBX5	MBX104
4-Way	Random mating 4-way R MBX5	MBX5	MBX105
4-Way	Random mating 4-way R MBX5	MBX5	MBX106
4-Way	Random mating 4-way R MBX5	MBX5	MBX107
4-Way	Random mating 4-way R MBX5	MBX5	MBX108
4-Way	Random mating 4-way R MBX5	MBX5	MBX109
4-Way	Random mating 4-way R MBX5	MBX5	MBX110
4-Way	Random mating 4-way R MBX5	MBX5	MBX111
4-Way	Random mating 4-way R MBX5	MBX5	MBX112
4-Way	Random mating 4-way R MBX5	MBX5	MBX113
4-Way	Random mating 4-way R MBX5	MBX5	MBX114

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
4-Way	Random mating 4-way R MBX5	MBX5	MBX115
4-Way	Random mating 4-way R MBX5	MBX5	MBX116
4-Way	Random mating 4-way R MBX5	MBX5	MBX117
4-Way	Random mating 4-way R MBX5	MBX5	MBX118
4-Way	Random mating 4-way R MBX5	MBX5	MBX119
4-Way	Random mating 4-way R MBX5	MBX5	MBX120
4-Way	Random mating 4-way R MBX5	MBX5	MBX121
4-Way	Random mating 4-way R MBX5	MBX5	MBX122
4-Way	Random mating 4-way R MBX5	MBX5	MBX123
4-Way	Random mating 4-way R MBX5	MBX5	MBX124
4-Way	Random mating 4-way R MBX5	MBX5	MBX125
4-Way	Random mating 4-way R MBX5	MBX5	MBX126
4-Way	Random mating 4-way R MBX5	MBX5	MBX127
4-Way	Random mating 4-way R MBX5	MBX5	MBX128
4-Way	Random mating 4-way R MBX5	MBX5	MBX129
4-Way	Random mating 4-way R MBX5	MBX5	MBX130
4-Way	Random mating 4-way R MBX5	MBX5	MBX131
4-Way	Random mating 4-way R MBX5	MBX5	MBX132
4-Way	Random mating 4-way R MBX5	MBX5	MBX133
BC_2	BC_1 x SPS	[(Hyb. 6) x SPS] #1 x SPS	H6-1
BC_2	BC_1 x SPS	[(Hyb. 6) x SPS] #2 x SPS	H6-2
BC_2	BC_1 x SPS	[(Hyb. 20) x SPS] #3 x SPS	H20-3
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #1 x SPS	H24-1
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #2 x SPS	H24-2
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #4 x SPS	H24-4
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #7 x SPS	H24-7
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #8 x SPS	H24-8
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #9 x SPS	H24-9

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #10 x SPS	H24-10
BC_2	BC_1 x SPS	[(Hyb. 24) x SPS] #12 x SPS	H24-12
BC_2	BC_1 x SPS	[(Hyb. 37) x SPS] #1 x SPS	H37-1
BC_2	BC_1 x SPS	[(Hyb. 37) x SPS] #4 x SPS	H37-4
BC_2	BC_1 x SPS	[(Hyb. 37) x SPS] #5 x SPS	H37-5
BC_2	BC_1 x SPS	[(Hyb. 37) x SPS] #7 x SPS	H37-7
BC_2	BC_1 x SPS	[(Hyb. 37) x SPS] #11 x SPS	H37-11
BC_2	BC_1 x SPS	[(Hyb. 46) x SPS] #4 x SPS	H46-4
BC_2	BC_1 x SPS	[(Hyb. 46) x SPS] #5 x SPS	H46-5
BC_2	BC_1 x SPS	[(Hyb. 46) x SPS] #6 x SPS	H46-6
BC_2	BC_1 x SPS	[(Hyb. 82) x SPS] #1 x SPS	H82-1
BC_2	BC_1 x SPS	[(Hyb. 82) x SPS] #3 x SPS	H82-3
BC_2	BC_1 x SPS	[(Hyb. 82) x SPS] #9 x SPS	H82-9
BC_2	BC_1 x SPS	[(Hyb. 82) x SPS] #11 x SPS	H82-11
BC_2	BC_1 x SPS	[(Hyb. 82) x SPS] #13 x SPS	H82-13
BC_2	BC_1 x SPS	[(Hyb. 100) x SPS] #3 x SPS	H100-3
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX168
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX169
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX170
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX171
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX172
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX173
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX174
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX175
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX176
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX177
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX178
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX179

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX180
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX181
4-Way	BCG x BCG (Random Mating)	Four-way R BCG (screened)	MBX182
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX2 x (MBX2,5,16,18,20)	MBX183
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX2 x (MBX2,5,16,18,20)	MBX184
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX5 x (MBX2,5,16,18,20)	MBX185
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX5 x (MBX2,5,16,18,20)	MBX186
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX16 x (MBX2,5,16,18,20)	MBX187
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX16 x (MBX2,5,16,18,20)	MBX188
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX16 x (MBX2,5,16,18,20)	MBX189
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX16 x (MBX2,5,16,18,20)	MBX190
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX18 x (MBX2,5,16,18,20)	MBX191
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX20 x (MBX2,5,16,18,20)	MBX192
Fitness	F_2 x F_2 (Random Mating Tent 1)	MBX20 x (MBX2,5,16,18,20)	MBX193
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX2 x (MBX2,5,16,18,20)	MBX194
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX2 x (MBX2,5,16,18,20)	MBX195
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX2 x (MBX2,5,16,18,20)	MBX196
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX5 x (MBX2,5,16,18,20)	MBX197
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX16 x (MBX2,5,16,18,20)	MBX198
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX16 x (MBX2,5,16,18,20)	MBX199
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX16 x (MBX2,5,16,18,20)	MBX200
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX16 x (MBX2,5,16,18,20)	MBX201
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX18 x (MBX2,5,16,18,20)	MBX202
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX18 x (MBX2,5,16,18,20)	MBX203
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX20 x (MBX2,5,16,18,20)	MBX204
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX20 x (MBX2,5,16,18,20)	MBX205
Fitness	F_2 x F_2 (Random Mating Tent 2)	MBX20 x (MBX2,5,16,18,20)	MBX206
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX5 x (MBX2,5,16,18,20)	MBX207

Type	Description (♀ x ♂)	Parents[a] (♀ x ♂)	Line Name
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX5 x (MBX2,5,16,18,20)	MBX208
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX16 x (MBX2,5,16,18,20)	MBX209
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX16 x (MBX2,5,16,18,20)	MBX210
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX18 x (MBX2,5,16,18,20)	MBX211
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX18 x (MBX2,5,16,18,20)	MBX212
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX20 x (MBX2,5,16,18,20)	MBX213
Fitness	F_2 x F_2 (Random Mating Tent 3)	MBX20 x (MBX2,5,16,18,20)	MBX214
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX2 x (MBX2,5,16,18,20)	MBX215
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX2 x (MBX2,5,16,18,20)	MBX216
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX5 x (MBX2,5,16,18,20)	MBX217
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX5 x (MBX2,5,16,18,20)	MBX218
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX16 x (MBX2,5,16,18,20)	MBX219
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX16 x (MBX2,5,16,18,20)	MBX220
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX16 x (MBX2,5,16,18,20)	MBX221
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX18 x (MBX2,5,16,18,20)	MBX222
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX18 x (MBX2,5,16,18,20)	MBX223
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX18 x (MBX2,5,16,18,20)	MBX224
Fitness	F_2 x F_2 (Random Mating Tent 4)	MBX18 x (MBX2,5,16,18,20)	MBX225
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX2 x (MBX2,5,16,18,20)	MBX226
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX2 x (MBX2,5,16,18,20)	MBX227
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX5 x (MBX2,5,16,18,20)	MBX228
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX16 x (MBX2,5,16,18,20)	MBX229
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX16 x (MBX2,5,16,18,20)	MBX230
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX16 x (MBX2,5,16,18,20)	MBX231
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX18 x (MBX2,5,16,18,20)	MBX232
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX18 x (MBX2,5,16,18,20)	MBX233
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX20 x (MBX2,5,16,18,20)	MBX234
Fitness	F_2 x F_2 (Random Mating Tent 5)	MBX20 x (MBX2,5,16,18,20)	MBX235

[a] MO1 females followed by a number or letter indicates that the female was a clone. SPS indicates smooth pigweed (susceptible to ALS). Males of the same type followed by the same number indicates that the same male was present in multiple crosses. C4 indicates plants from the fourth generation of the recurrent selection for glyphosate resistance project. Fitness indicates F_2 plants randomly mated for use in a fitness penalty study. Plants were not selected for any types of resistance, but ALS, PPO, triazine, and glyphosate resistance should be present at some level in each of the seed lines.

APPENDIX C

HERBICIDE AND LIQUID FERTILIZER MIXING INSTRUCTIONS

C.1 Introduction

The purpose of this appendix is to aid applicators in mixing liquid fertilizer, as well as in mixing the herbicides that I have often applied in the past. I have already done the work of calculating the appropriate amount of herbicide to add in a mixture, and although such an exercise would be instructive for the applicator to perform, the following is meant to be a time-saving tool in mixing.

C.2 Essential Information for Herbicide Mixing

The moving-nozzle cabinet sprayer should be calibrated to deliver 187 L ha^{-1} of spray solution. However, when reading herbicide labels, rarely do they refer to spray volume in L ha^{-1}; it is practically always referred to in gallons per acre (gallons $acre^{-1}$). In these units, the sprayer should be calibrated to deliver 20 gallons $acre^{-1}$.

Also it is important to keep in mind that the sprayer platform can hold approximately 40 4.5” square pots when plants are small (5 cm stage). However, as the plants grow (up to 10–15 cm in height), their leaves tend to extend past the boundaries of the pots, and in order to ensure adequate spray coverage of each plant at this stage, a more realistic estimate is that the platform will hold approximately 20 pots. The sprayer is calibrated to spray 40 mL of spray solution per pass. This corresponds to the entire volume of the vial used for spraying when filled up to the point at which the neck begins to narrow. This information will help for planning the amount of herbicide needed to treat the plants in a particular study.

C.3 Sprayer Operation

The applicator should wear nitrile gloves while spraying. The spray chamber operates via air pressure, and thus the airflow must be turned on by opening a valve (Figure C.1) before use. Once airflow has been turned on, the sprayer should be rinsed twice with water by first filling an empty 40 mL vial with water and inserting it into the stainless steel sleeve (Figure C.2). (Note: two steel sleeves are present near the sprayer. Only the larger of the two actually fits well enough to form an airtight seal, and thus this is the one that should be used.) The stainless steel tube should then be inserted in the sprayer. Often the first time this is done during spraying, the fit may be extremely tight. Thus, it is best to wet the rubber washers that the sleeve pushes against with your fingers before trying to force the sleeve into its housing. Once the sleeve has been fitted into the housing in the sprayer, the doors should be closed by pushing the "Close" button (Figure C.3).

After the doors have been closed, push the "Right" button to spray the first water rinse. When the nozzle has reached the right end of the track, push the "Stop" button to stop the flow through the nozzle. Then push "Left" to return the nozzle to its original position. At this time, the doors can be opened again by pushing the "Open" button. The stainless steel sleeve should then be removed and the 40 mL vial refilled with water, and this process should then be repeated to complete the second rinse.

Afterward, the spray chamber is ready for spraying. At this time, the platform should be raised (before loading it with plants) to a height at which the plant canopy will be approximately 18 inches (45 cm) below the nozzle. To raise the platform, press the "Raise" button. Next, the platform can be loaded with plants, with care taken to ensure that leaves from separate plants do not overlap one another. At this point it is recommended that the distance between the nozzle and

the plant canopy be checked to ensure that the distance is approximately 45 cm. If necessary, the platform can be lowered by pressing the "Lower" button. The steel sleeve should then be removed, and any residual water poured from the vial. The vial should then be filled with herbicide and reinserted into the sleeve, which should be placed back into its housing in the spray chamber to begin spraying. The subsequent spraying process is performed similarly to the initial rinsing process.

When spraying multiple doses of the same herbicide, it is recommended that the applicator begin with the lowest doses and work up to higher doses to prevent any residual herbicide left in the sprayer from causing undesired consequences to the next group of plants to be sprayed. If different herbicides are being used, it is recommended that the sprayer be rinsed twice with water as described above between different herbicide applications to remove residual herbicide from the sprayer. Leftover herbicide solutions should be disposed of in the herbicide disposal container located on the floor to the left of the spray chamber.

It is important that the spray chamber be properly cleaned after use. To clean the sprayer, first fill a 40 mL vial with a 1:1 mixture of water and ammonia. This solution should be sprayed to neutralize herbicide in the tubing, the nozzle, and on the surfaces of the spray chamber. Next, the sprayer should be rinsed by spraying two vials of water. After rinsing with water, any residual soil should be wiped from the platform. Residual herbicide tends to pool on the floor of the spray chamber, and this should be absorbed with paper towels. Finally, all surfaces of the spray chamber, including the platform, the inside of the glass doors, the walls, and the floor should be sprayed with a 1:1 solution of ammonia and water from the spray bottle located near the sink, and one of the two squeegees should then be used to wipe all surfaces clean. Finally, the platform should be lowered, the stainless steel sleeve should be removed from the sprayer, the

doors should be closed, and the airflow should be turned off. In this way, the spray chamber will be made ready for use by the next applicator.

C.4 General Calculation

When calculating the amount of herbicide to add to a mix, the calculation should begin with the desired dose and should then proceed to the required amount of herbicide to add via dimensional analysis. Oftentimes the herbicide label will suggest a dose in terms of oz acre^{-1} or pounds acre^{-1}. However, as lab equipment uses SI units for measurement, ultimately the calculated amount of herbicide to use should be in units of mL (or µL) or g, respectively. Thus, calculations for the amount of herbicide to use will have to take into account the appropriate conversions. Several example calculations follow.

C.4.1 Spraying WeatherMAX

As one example, assume that 80 plants at the 10–15 cm stage are to be sprayed with the regular field use rate of Roundup WeatherMAX® (22 oz acre^{-1} according to the label). Since these plants are likely to have leaves extending over the edges of the pots, the applicator should plan for spraying only 20 plants per pass, indicating that at least four passes will be required to treat all 80 plants. At 40 mL per pass, this amounts to 160 mL required, and it would be wise to mix enough solution for 5 sprays in case of problems, in which case the total spray volume required becomes 200 mL. Ultimately then, the applicator is interested in determining how much of the liquid WeatherMAX to add to the 200 mL spray solution. A sample calculation is provided below, in which the basic strategy is to convert from the desired dose of 22 oz acre^{-1} to a dose in terms of mL weathermax in 200 mL of spray solution. Using the information given

above, as well as standard unit conversions, the applicator will have enough information to solve this problem.

22 oz WM	2.47 acres	1 ha	1 L spray solution	29.6 mL WM	200 mL spray solution	=	1.720 mL WM to add to 200 mL spray
1 acre	1 ha	187 L spray solution	1000 mL spray solution	1 oz WM			

The applicator would also add 200 mL x 2.5% (v/v) AMS = 5 mL AMS to this solution.

Many scientific papers refer to glyphosate doses in g ae ha^{-1}. In this case, if it is assumed that the applicator needs to apply glyphosate at 840 g ae ha^{-1} (the regular field use rate), the calculation for the amount of WeatherMAX to use is similar to that shown above:

840 g ae	1 ha	1 L spray solution	1 L WM	1000 mL WM	200 mL spray solution	=	1.664 mL WM to add to 200 mL spray
1 ha	187 L spray solution	1000 mL spray solution	540 g ae	1 L WM			

where in this calculation, the fact that 1 L of WeatherMAX contains 540 g ae glyphosate was used. The amount of glyphosate added to the 200 mL mix is slightly different between the two calculations—this difference (56 µL) is attributable to the fact that 840 g ae ha^{-1} does not correspond exactly to 22 oz $acre^{-1}$. However, as the 1x dose of glyphosate (in terms of g ae ha^{-1}) varies in the literature, I have consistently used 840 g ae ha^{-1} as the 1x dose in all of my work.

As the previous examples have demonstrated calculations for the use of a liquid herbicide, the following example demonstrates the same type of calculation performed for a solid herbicide.

C.4.2 Spraying atrazine

For the following example, again assume that 80 plants at the 10–15 cm stage are to be sprayed with the regular field use rate of Aatrex Nine-O (1000 g ai ha^{-1}). Again, due to plant size, the applicator should plan for spraying only 20 plants per pass, and should mix enough herbicide for five passes in case of problems, meaning that 200 mL of spray solution will be required. Thus, the applicator is interested in determining how much atrazine to add to the 200 mL spray solution. A sample calculation is provided below for determining the amount of product required.

1000 g ai	1 ha	1 L spray solution	1 g Aatrex	200 mL spray solution	=	1.188 g Aatrex in 200 mL spray solution
1 ha	187 L spray solution	1000 mL spray solution	0.9 g ai			

Here, the fact that Aatrex Nine-O consists of 90% active ingredient by weight has been used to convert from g ai to g product to be measured. The user would also add 1% (v/v) COC to this mixture, giving 200 mL x 1% COC = 2 mL COC. Similar calculations can be performed for all herbicides. However, I have found that simplification of these calculations can save some time and lead to increased confidence in proper mixing of herbicides. For instance, the above calculation for the application of atrazine could be simplified by combining the constants, but

leaving the dose in g ai ha^{-1} as well as the total amount of spray solution to mix as variables. Combining the remaining constants gives 1/168,300, and the resulting equation with variables becomes

$$\text{Amount of Aatrex Nine-O needed (g)} = \frac{\text{Dose (g ai ha}^{-1}\text{)} \times \text{Amount of spray solution needed (mL)}}{168{,}300}$$

A sample calculation using this equation for the situation above (spraying 80 plants at the 10–15 cm stage with atrazine at 1000 g ai ha) follows.

$$\text{Amount of Aatrex Nine-O needed (g)} = \frac{\text{1000 g ai ha}^{-1} \times \text{200 mL spray solution}}{168{,}300} = \text{1.188 g Aatrex Nine-O}$$

C.5 Reduced Mixing Equations for Various Herbicides

C.5.1 Weathermax

This herbicide is formulated at 540 g ae per liter. Include 2.5% (v/v) AMS when spraying this herbicide.

C.5.1.1 Dose in terms of g ae ha^{-1}

$$\text{Amount of Weathermax needed (mL)} = \frac{\text{Dose (g ae ha}^{-1}\text{)} \times \text{(mL spray solution)}}{100{,}969}$$

C.5.1.2 Dose in terms of x (times the field use rate)

$$\text{Amount of Weathermax needed (mL)} = \frac{\text{Dose (x field use rate)} \quad X \quad \text{(mL spray solution)}}{116.392}$$

C.5.2 Touchdown Hi-Tech

This herbicide is formulated at 5 lb ae per gallon. Include 2.5% (v/v) AMS and 0.25% (v/v) NIS when applying this herbicide.

$$\text{Amount of Touchdown needed (mL)} = \frac{\text{Dose (g ae ha}^{-1}\text{)} \quad x \quad \text{(mL spray solution)}}{112{,}039}$$

C.5.3 MON76255

This herbicide is formulated at 0.556 g ae per mL. Include 2.5% (v/v) AMS and 0.25% (v/v) NIS when applying this herbicide. The 1x dose is calculated assuming an application rate of 840 g ae ha^{-1}.

$$\text{Amount of MON76255 needed (mL)} = \frac{\text{Dose (x field use rate)} \quad x \quad \text{(mL spray solution)}}{122.306}$$

C.5.4 Aatrex Nine-O

Include 1% (v/v) COC when making foliar applications of this herbicide.

$$\text{Amount of Aatrex needed (g)} = \frac{\text{Dose (g ai ha}^{-1}\text{)} \quad x \quad \text{(mL spray solution)}}{168{,}300}$$

C.5.5 Blazer

This herbicide is formulated at 2 lbs ai per gallon. Include 1% (v/v) COC when applying this herbicide.

$$\text{Amount of Blazer needed (mL)} = \frac{\text{Dose (g ai ha}^{-1}\text{)} \quad X \quad \text{(mL spray solution)}}{44{,}816}$$

C.5.6 Cobra

This herbicide is formulated at 2 lbs ai per gallon. Include 1% (v/v) COC when applying this herbicide.

$$\text{Amount of Cobra needed (mL)} = \frac{\text{Dose (g ai ha}^{-1}) \quad \text{x} \quad \text{(mL spray solution)}}{44{,}816}$$

C.5.7 Raptor

This herbicide is formulated at 1 lb ai per gallon. Include 1% (v/v) COC and 2.5% (v/v) AMS when applying this herbicide.

$$\text{Amount of Raptor needed (mL)} = \frac{\text{Dose (g ai ha}^{-1}) \quad \text{x} \quad \text{(mL spray solution)}}{22{,}408}$$

C.5.8 Pursuit 70DG

This herbicide is composed of 70% ai by weight.

$$\text{Amount of Pursuit needed (g)} = \frac{\text{Dose (x field use rate)} \quad \text{x} \quad \text{(mL spray solution)}}{1870.2}$$

C.6 Mixing Liquid Fertilizer

At times I have found it useful to apply liquid fertilizer to plants—particularly waterhemp seedlings, which generally must be watered from a watering can to prevent injury to the seedlings. During such waterings, liquid fertilizer can easily be added to the water in the can to fertilize the seedlings. I aimed for a fertilizer concentration of 200 ppm nitrogen during such applications. To do this, I began by dissolving 40 g of 20-20-20 Peters brand fertilizer in 100 mL of water. To apply this mixture then, I poured approximately 13 mL in 1 gallon of water in the watering can, to reach the desired concentration of approximately 200 ppm.

C.7 Figures

Figure C.1 Valve controlling airflow to spray chamber. Valve should be in the upright position when closed and in the horizontal position when open.

Figure C.2 Stainless steel sleeve. 40 mL tube should be inserted into the longer of the two sleeves present near the spray chamber before spraying.

Figure C.3 Spray chamber. All buttons but the “Spray” button are effective.